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March 1996

U.S. Dairy Forage Research Center 1995 Research Summaries

U.S. Dairy Forage Research Center. 1996. U.S. Dairy Forage Research Center 1995 Research Summaries. U.S. Department of Agriculture, Agricultural Research Service, 122 pp.

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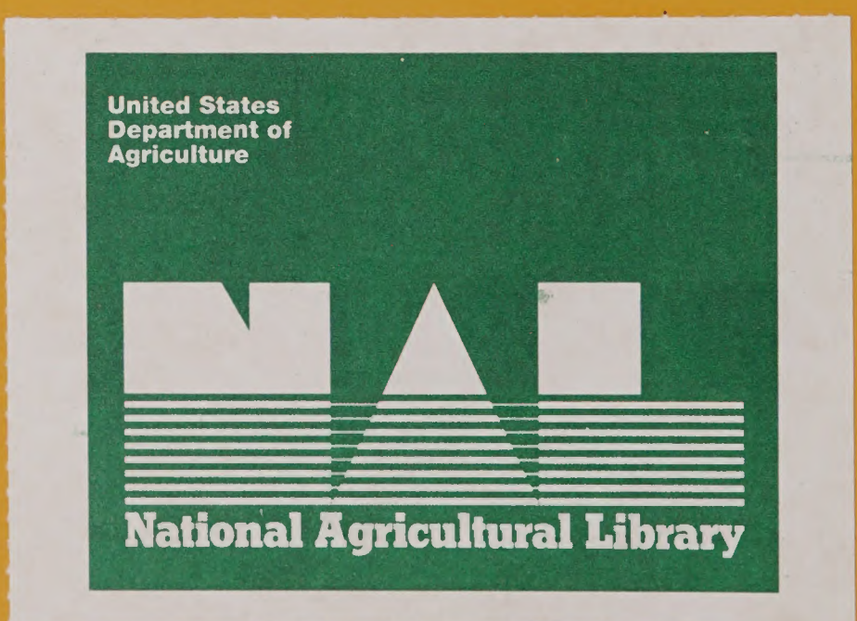
Appreciation is expressed to Gloria Palmer for her interest in and dedication to the task of typing and assembling this annual research summary.

Issued March 1996



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PLANT CHEMISTRY

RUMEN MICROBIOLOGY

FEED UTILIZATION BY CATTLE

Preface

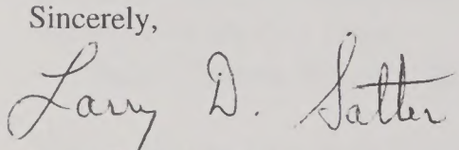
It is a pleasure to update our progress by bringing you these summaries of recent research. The U.S. Dairy Forage Research Center (USDFRC) is a unique part of the national research program of the Agricultural Research Service, U.S. Department of Agriculture. The Center's mission is to build a knowledge and technology base for the dairy industry to fully exploit the use of forages in the production of milk. The Center has agricultural engineers, plant and soil scientists, microbiologists, ruminant nutritionists and a chemist working together to increase the efficiency of forage production and utilization by dairy farmers. We function in close cooperation with the Agricultural Experiment Stations of several states. The Center is located on the campus of the University of Wisconsin, Madison, and has "Cluster" locations in St. Paul, MN, Ames, IA, East Lansing, MI, and Ithaca, NY. The Center's research farm with facilities for 330 milking cows is located on 63 acres of USDA land on the banks of the Wisconsin River in Prairie du Sac, WI. An additional 1200 acres of adjacent land is utilized by the Center by agreement with the U.S. Department of the Army. The Center was established in 1980 and has made steady growth since. At present there are fifteen scientists: ten at Madison, and one at each of three Cluster locations, and two at the St. Paul, Minnesota Cluster location. Scientists hold faculty appointments in university departments and provide supervision for approximately 15-20 graduate students and 5-10 post doctoral fellows.

Last year in this report we highlighted the continuing development of DAFOSYM, a computer systems model that simulates the operation of a dairy farm. DAFOSYM is the focal point of the systems work group within the USDFRC, and we feel it is a tool that can be very useful in making strategic management decisions on the dairy farm. This year in our report we are highlighting USDFRC research that relates to protein utilization by the dairy cow. The protein contained in most forage plants is very easily degraded by rumen microbes, and this can result in poor utilization of forage protein by the cow. It can also contribute to excess nitrogen in animal manure and to risk of environmental pollution. Research at USDFRC is attacking this problem from several approaches, and our progress is reported.

We are planning a USDFRC Informational Conference September 16-18, 1996 in Madison. The objective is to foster communication between USDFRC and the dairy and forage industry. The conference will be conducted in sessions over two and a half days. Presentations will be given by members of the USDFRC staff followed by questions/discussion. In addition, a poster session will be held to provide additional information concerning specific research projects at the USDFRC. If you are interested in attending, please contact us.

We are pleased and very proud of the way Center scientists from diverse disciplines interact and bring their collective insights to bear on the problems of forage production and utilization. This collection of research summaries illustrates the progress they are making in developing information to help dairy farmers utilize their resources more effectively. The research is intended to benefit producers of forage crops, dairy farmers and the consumers of dairy products.

Sincerely,



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Protein Nutrition Research at the U.S. Dairy Forage Research Center

Forages help meet the protein requirements of dairy cows by providing N as ruminally degraded protein for microbial protein formation plus protein that escapes degradation in the rumen. Evidence from many feeding studies with lactating cows indicates that too much protein in legume and grass forages is lost in the rumen. This problem has two major ramifications: 1) the cost of supplemental protein to compensate for the high ruminal losses of forage protein; and 2) excessive N excretion from the cow that contributes to environmental pollution. A number of projects at the Dairy Forage Center are addressing this problem.

Reducing Nonprotein Nitrogen in Silages Will Improve Their Protein Value

Ensiling reduces the value of protein in forage. During ensiling, plant cells are broken, releasing enzymes that break down proteins into nonprotein nitrogen (NPN). In alfalfa, NPN typically accounts for 50 to 65% of silage crude protein. This loss of true protein reduces N utilization by the cow. Feeding trials at the Center's Research Farm demonstrated that cows fed alfalfa silage with lower NPN and higher true protein produced more milk and protein than cows fed alfalfa silage with more NPN, even though crude protein content of both diets was equal. More of the dietary N went into milk and less went into manure. Reducing N loss in manure would be friendlier to the environment, but the farmer also has an economic incentive for improving N efficiency. An analysis using DAFOSYM, the Center's whole-farm economic model, indicated that an average dairy farm in the northern U.S. loses up to $28/acre each year from breakdown of alfalfa protein in the silo. Figure 1 shows the NPN content of silages made from several legume forages. Of the legumes tested, alfalfa had the worst problem with NPN. Legumes containing tannins had reduced silage NPN; the reduction was related directly to tannin level. Of the forages without tannins, cicer milkvetch and particularly red clover had lower NPN; apparently these forages have alternative mechanisms to reduce silage NPN.

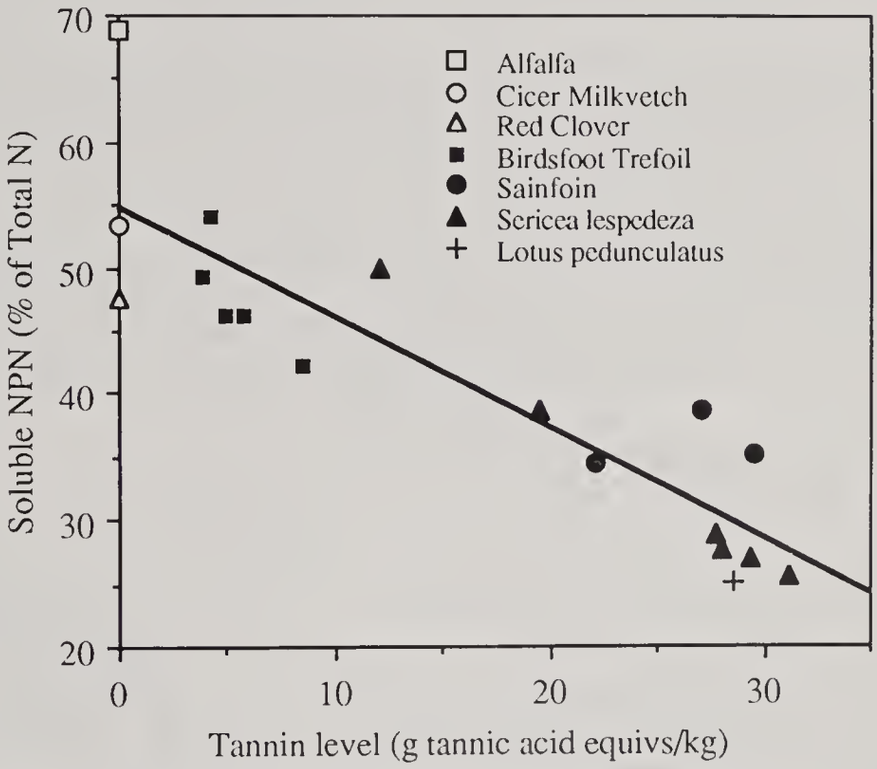

Figure 1. Soluble nonprotein N (NPN) content of silages made from legumes with varying tannin contents.

An Unusual Enzyme in Red Clover Improves Its Protein Value

Red clover, a forage with similar protein content to alfalfa, forms up to 90% less NPN than alfalfa during ensiling. This suggests that red clover is a better legume to ensile. Yet the widespread use of red clover is limited by its lower yield, low field stand persistency, and slow drying rate in the field. Research was undertaken on red clover to determine the mechanism for its lower proteolysis during ensiling. Proteolysis was measured as free amino acid release in soluble extracts from alfalfa and red clover (Figure 2). After 4 hours, proteolysis was six times greater in alfalfa extract than in red clover extract; mixing alfalfa and red clover extracts resulted in proteolysis about equal to red clover alone. Boiling (which destroys enzymes) the red clover extract before adding it to alfalfa extract actually increased proteolysis over alfalfa alone. Adding ascorbate, which interferes with the action of polyphenol oxidase (PPO), increased red clover proteolysis to about 40% of alfalfa. Red clover contains several phenols, including caffeic acid, that PPO

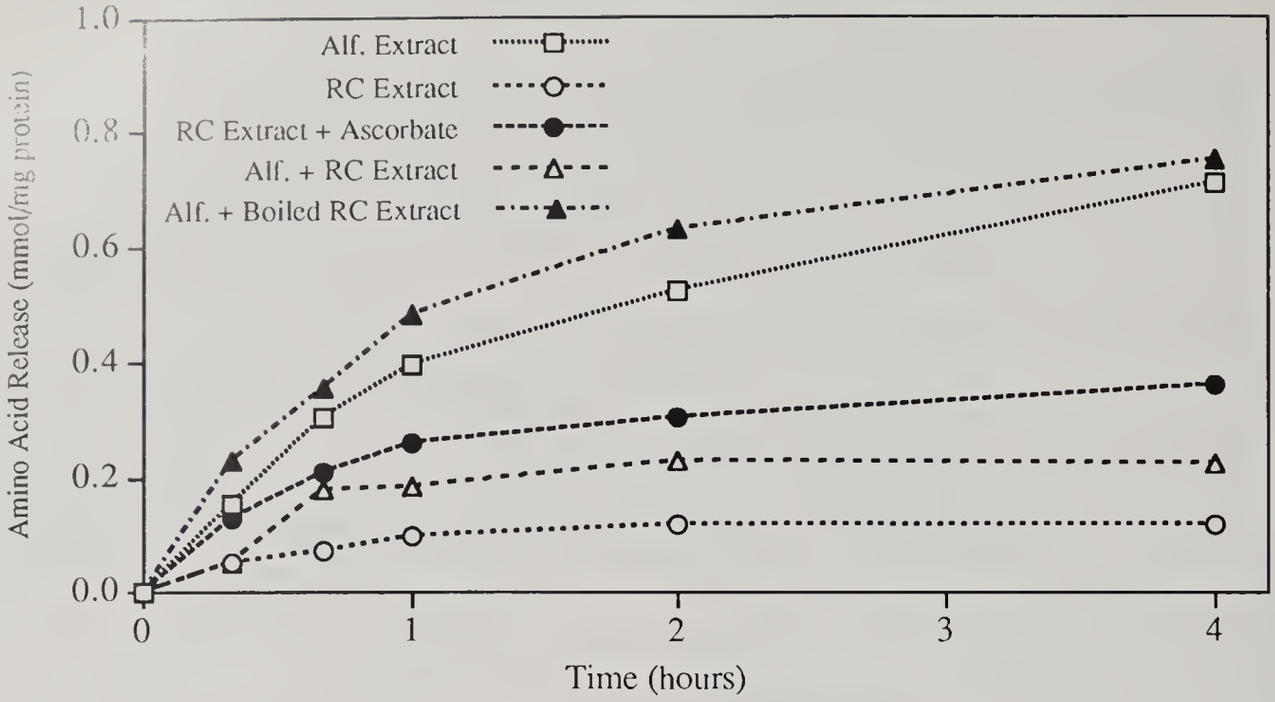

Figure 2. Proteolysis in extracts from alfalfa (Alf.) and red clover (RC) forages.

uses to form quinones; quinones react very rapidly with proteases, the enzymes that break down proteins. During incubation with red clover extracts, caffeic acid and two other as yet unidentified phenols decrease along with the decrease in proteolytic activity. These and other findings proved that the reduced proteolysis in red clover is not due to inherent differences in proteolytic activity between red clover and alfalfa but occurs because the PPO in red clover produces quinones that react with the proteases.

Alfalfa Hay Has Greater Protein Value Than Alfalfa Silage

Lower NPN content of alfalfa hay makes it a better protein source for the cow than alfalfa silage. Lactating cows were fed all their forage as either silage or alfalfa hay in two studies at the Center's Research Farm. Alfalfa was harvested from alternate windrows as either silage with 40% DM or as hay in small bales. Silage averaged 20.6% crude protein and hay 18.1% crude protein. Alfalfa hay had lower protein because of greater leaf loss during harvest. The NPN contents were 52% (silage) and 8% (hay) of total N. Diets contained(DM basis) 67% alfalfa, 32% high moisture ear corn, and 1% minerals and vitamins. Two diets were supplemented with 3% fish meal; greater milk and protein yield with fish meal would mean that the diet without fish meal had a lower protein value.

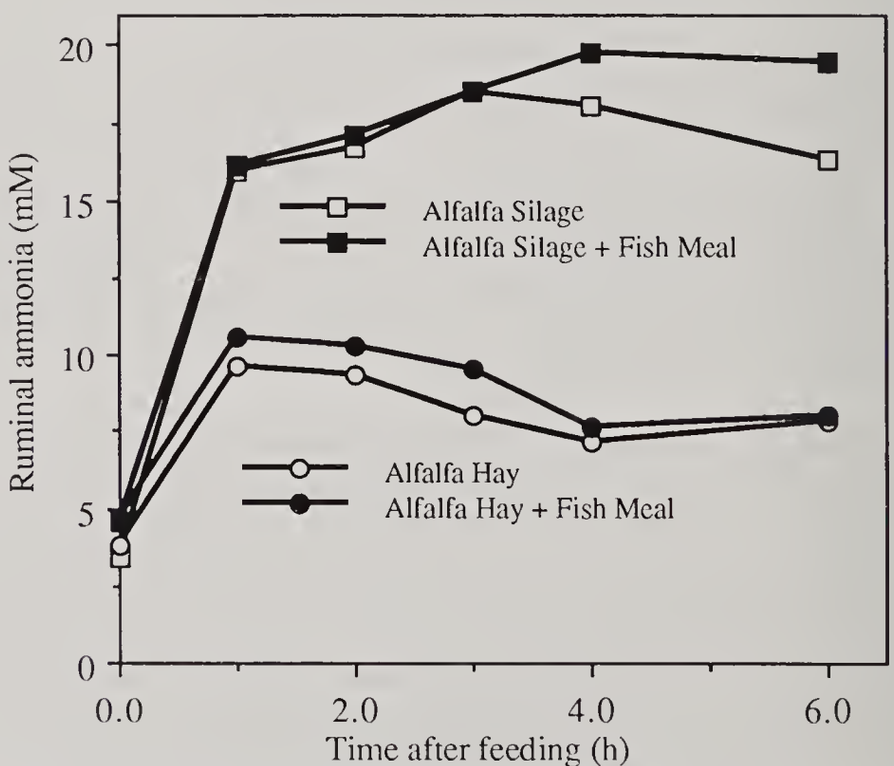

Figure 3. Ruminal ammonia levels in dairy cows fed all their forage as either alfalfa silage or alfalfa hay, with or without supplemental fish meal.

Yields of milk, protein and solids-not-fat were higher on alfalfa hay than on silage without fish meal. Feeding fish meal increased milk and protein yield 2.1 kg/d (4.5 lbs/d) and 100 g/d on silage and .8 kg/d and 30 g/d on hay. Digestibility of DM in silage was greater than hay; this may be why there was increased milk yield after correction of protein inadequacy by adding fish meal. Ruminal ammonia, a good index of excessive breakdown of dietary crude protein (Figure 3), clearly was greater at all times after feeding silage, averaging 15.4 mM (silage) and 8.1 (hay). Some in vitro results not shown here indicated that the ruminal microbes made more protein out of the degraded protein in hay than in silage. New developments in mechanization may improve hay harvesting. Koegel and his colleagues in the Center's engineering group have developed an alternative hay-making process involving extensive shredding of the forage prior to forming it into thin forage mats and field-drying. Drying time for the shredded alfalfa was only one-third that for conventionally harvested alfalfa hay. The energy and protein values of the shredded hay also were greater.

Grain Processing to Improve Ruminal Microbial Protein Formation

Yet another approach to the problem of excess NPN in alfalfa silage is enhancing N capture for ruminal microbial protein. Grinding corn to particle sizes of 600 to 700 microns improves ruminal digestion, thus promoting microbial growth and utilization of degraded protein and NPN. Some experiments at the Center demonstrated that grinding of high moisture corn through a 3/8" screen depressed ruminal ammonia (Figure 4), a measure of excessive protein degradation, and improved milk yield by 2.0 kg/day (4.4 lbs/d) over feeding the same amount of unground high moisture corn. In vitro tests with this and more finely ground high moisture corn indicated that finer grinds would not further increase microbial protein synthesis. Studies are continuing to see how much the protein supplement can be reduced by finely grinding the corn.

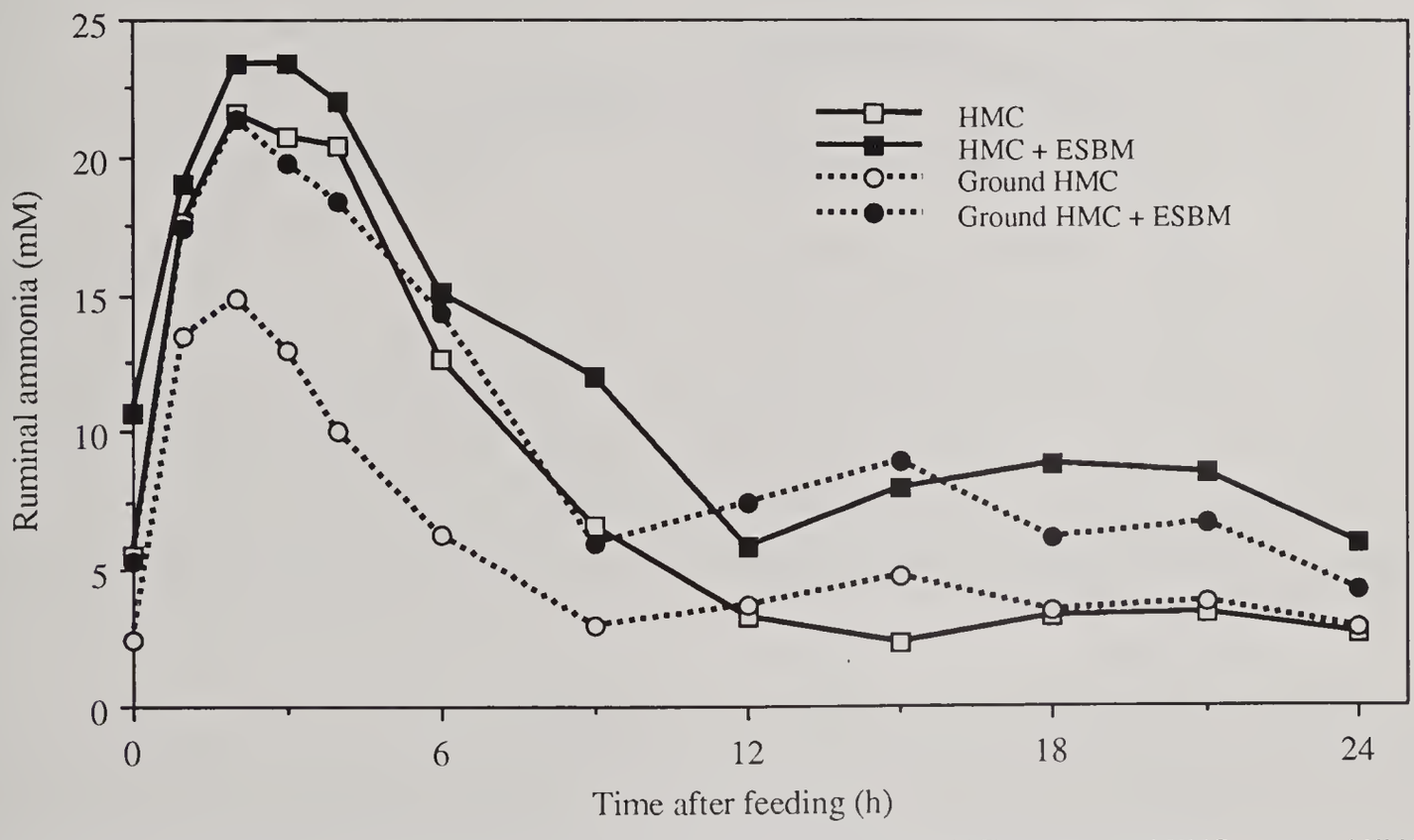

Figure 4. Ruminal ammonia concentrations in dairy cows fed high moisture corn (HMC) as unground HMC or ground HMC in alfalfa silage diets. A high bypass protein, expeller soybean meal (ESBM) was fed in one diet with each HMC.

Heat Processing of Soybeans Improves Their Protein Value

Another way to approach the problem of excessive degradation of forage protein is to reduce degradation of the other proteins fed to the dairy cow. Soy protein is the primary source of supplemental protein for dairy

cattle in the U.S. and can be fed as soybean meal or as full fat soybeans. With conventional soybean meal, about 30-35% of the protein will escape degradation in the rumen. With soybeans, only about 25% escapes. Heat treatment can increase ruminal escape for soybeans to 50 to 60%. Research at the Center has provided the information needed to optimize the heat treatment of full fat soybeans and, further, has demonstrated the milk production response that can be obtained with properly heated soybeans. Figure 5 illustrates the improvement obtained when conventional soybean meal, unheated (Raw) soybeans and "properly" roasted soybeans were fed to lactating cows. Feeding roasted soybeans improved milk yield an average of 4 kg/day (9 lbs/day) compared to feeding the same amount of protein as soybean meal or unheated soybeans. Protein dispersibility index (PDI), an existing test for estimating extent of heat exposure, was standardized in laboratory and animal studies at the Center using numerous batches of roasted soybeans, from raw to extensively overheated. The PDI test, properly standardized for roasted soybeans, is now used in commercial labs for assessing the quality of heated soybeans. The availability of properly roasted soybeans and a test to assure their quality, have been instrumental in the growth of heat processing of soybeans for feeding to dairy cows in the U.S. from negligible amounts in 1985 to current usage of approximately 20 million bushels per year.

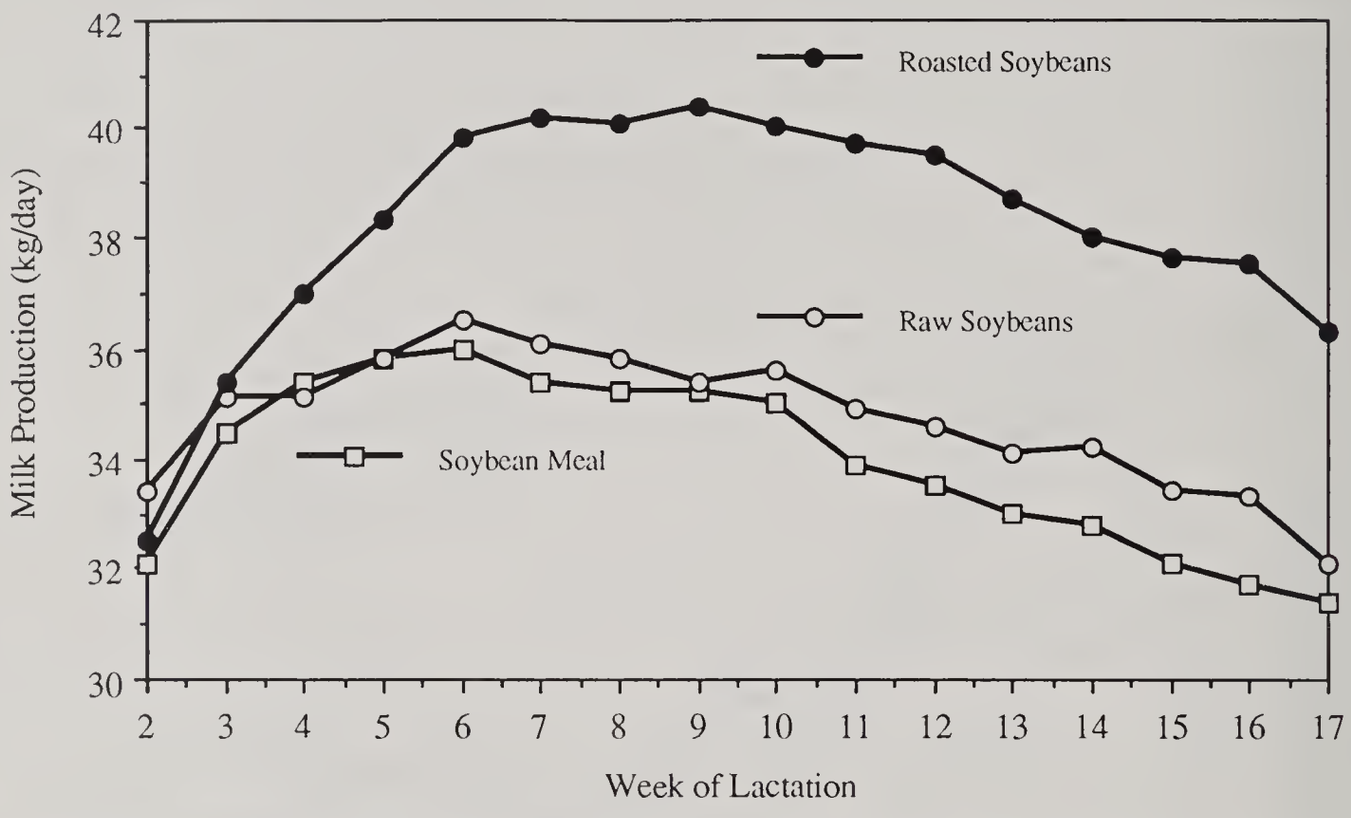

Figure 5. Milk yield with feeding equal amounts of supplemental protein as soybean meal, unheated (raw) soybeans or "properly" roasted soybeans to lactating cows.

Protein Nutrition Research and the Mission of the Center

The mission of the U.S. Dairy Forage Research Center is to apply the tools of basic and applied science to improving forage utilization by dairy cows. Forages are one of the principal protein sources in dairy cattle rations. Excess ruminal degradation of forage protein makes protein the nutritional factor that most limits the value of high quality forages by lactating cows. The Center's continuing work on protein nutrition is finding ways to improve utilization of forage protein and is putting the tools to accomplish this task into the hands of the Nation's dairy farmers.

Recurrent Phenotypic Selection for Seed Size in Kura Clover

R.R. Smith

Introduction

Kura clover (*Trifolium ambiguum* M. Bieb.) evolved in the cool, temperate environments in the Caucasian regions of Central Asia. This perennial legume is rhizomatous, drought tolerant, winterhardy and persistent—desirable attributes for a pasture and soil conservation legume in many of the temperate regions of the world. Established kura clover persists under frequent defoliation, has excellent forage quality and is extremely winterhardy. However, a major disadvantage of kura clover is poor or slow initial establishment. Increasing seed size in other forage legumes has been successful in enhancing initial establishment. This paper reports on the response in the diploid (2x), tetraploid (4x) and hexaploid (6x) forms of kura clover to two cycles of phenotypic selection for seed size and to the influence that increased seed size had on the initial establishment of the hexaploid form.

Materials and Methods

Selection procedures. The base populations (Cycle 0) used for the initial selection of large seed were plant introductions provided by the National Plant Germplasm System. Plant Introductions 225828 and 225827 represented the diploid (2x) form, 108699, 258787, 405119, 405120, and 405121 the tetraploid (4x) form and 258788, 405122, 405123, 405124, 440703 and 440714 the hexaploid (6x) form. Seed was saved that would not pass through 1.6, 1.7 and 1.8 mm diam. metal sieves for the 2x, 4x and 6x forms, respectively. Seventy plants from the large seed lots in each ploidy form were intercrossed to produce cycle 1 (C1) seed. This C1 seed was screened, and seed was saved that did not pass through 1.7, 1.8 and 1.9 mm diam. metal sieves for the 2x, 4x and 6x forms, respectively. Sixty-five large-seeded C1 plants were intercrossed to produce cycle 2 (C2) seed. Finally, to avoid the influence of environment on seed size, fifty representative plants in each cycle and each ploidy level (9 populations) were intercrossed in one environment, the Agric. Res. Sta., Arlington, WI. Honeybees (*Aphis* spp.) were used as the pollinator. Seed size is reported as seed weight in grams per 1000 seed.

Agronomic evaluations. To evaluate the influence of increased seed size on seedling establishment and vigor, the cycle 0 and cycle 2 populations of the hexaploid form were established in 0.9 x 7.5 m five row plots on the Agric. Res. Sta., Arlington, WI in May, 1994 and 1995. Seeding rate was approximately 500 plants m^{-2}. Seedling emergence (plants m^{-2}) was determined 8 wk after planting, forage yield (Mg dry matter ha^{-1}) was measured on 15 Aug. 1994 and 25 Aug. 1995, and visual estimates of the degree of ground cover (% stand) recorded 2 weeks after harvest. In 1995, two harvests were taken (20 Jun. and 8 Aug.) on the 1994 established study.

Results and Conclusions

Recurrent phenotypic selection for seed size in kura clover was effective in all three ploidy levels. Seed size increased from 1.23 g per 1000 seeds in the C0 to 1.84 g in the C2 for the diploids, from 1.81 g to 2.02 g in the tetraploids, and from 1.92 to 2.33 g in the hexaploids (Fig. 1). The largest increase per cycle was detected in the diploid population (0.36 g

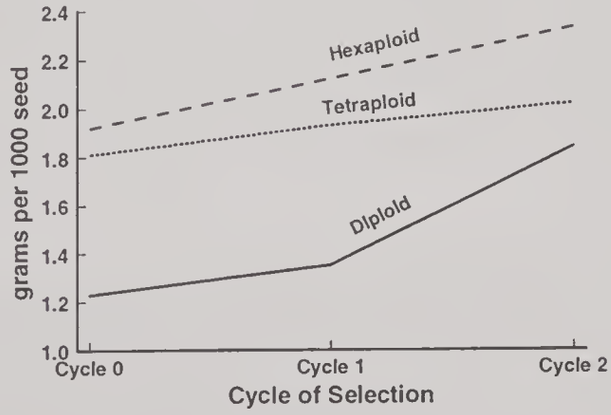

Figure 1. Response of diploid, tetraploid and hexaploid kura clover to recurrent selection for seed size.

per 1000 seeds) in contrast to 0.11 g for the tetraploids and 0.22 g for the hexaploid.

Averaged over two seedling year tests, the larger seeded hexaploid population (C2) had improved seedling emergence (265 vs. 177 seedlings m^{-2}), higher forage yield (1.23 vs. 0.95 Mg ha^{-1}) and percent ground cover (83 vs. 52) in contrast to the original population (C0) (Table 1).

The higher forage yield (6.0 Mg ha^{-1}) of the large seeded hexaploid population in the second year of the 1994 established test reflects the advantage this population had in the seedling year in contrast to the original population (Table 1).

Recurrent phenotypic selection was effective in increasing seed size in kura clover and appeared to have an influence on seedling establishment and subsequent forage production. Further selection appears to be possible and should further improve seedling establishment.

Table 1. Seedling emergence, forage yield and percent ground cover of hexaploid kura clover selected for seed size.

	Emergence			Yield			Percent stand		
	1994	1995	Avg.	1994	1995	Avg.	1994	1995	Avg.
Seedling year data									
	plts/ m sq			Mg/ ha			%		
Cycle 0	144	210	177	0.33	1.57	0.95	36	68	52
Cycle 2	289*	240	265*	0.67*	1.79	1.23*	83*	84*	83*
Forage yield (Mg per ha) for 1994 seeded test									
	Cut I	Cut II	Total						
Cycle 0	3.85	1.10	4.95						
Cycle 2	4.74*	1.26*	6.00*						

*Differences between cycles significant at the 5% level of probability.

Germplasm Effects on Subsoil Nitrate Uptake by Alfalfa

J.M. Blumenthal, M.P. Russelle and J.F.S. Lamb

Introduction

Excessive N applications as commercial fertilizer, animal manure, and organic waste products have been implicated in contamination of surface water, ground water, and the atmosphere. Field studies routinely show that soil inorganic N concentrations and nitrate leaching losses are smaller under deeply rooted perennial crops, like alfalfa (*Medicago sativa* L.), than under annual crops, like corn (*Zea mays* L.).

Nitrogen removed in alfalfa herbage consists not only of N from the inorganic soil N supply, but also atmospheric N_2 fixed by *Rhizobium meliloti* in root nodules and N remobilized internally from crowns and roots. In an earlier study we discovered that ineffective, non-N_2-fixing alfalfa was more efficient in subsoil nitrate removal than its N_2-fixing parent. Nutrient uptake often is related to root system architecture, and recent development of alfalfas that have divergent root architecture traits provides a means to test this in alfalfa. In addition, nitrate is reduced in both the roots and leaves of alfalfa, so the multileaf trait found in many commercial cultivars may provide an advantage for nitrate assimilation.

Our objectives were to: 1) reevaluate the efficacy of ineffective alfalfa at absorbing subsoil nitrate; and 2) compare removal of subsoil nitrate in herbage of several N_2-fixing alfalfa cultivars differing in their root system architecture and leaf morphology.

Materials and Methods

The experiment was conducted at the University of Minnesota Sand Plain Research Farm, Becker, MN, on a Hubbard loamy sand soil (sandy, mixed Udorothentic Haploboroll), which is underlain by gravel at 95-110 cm. Soaker hoses were buried in parallel about 30 cm apart and 50 cm deep in April 1994 before fertilizing and liming the topsoil according to University of Minnesota recommendations.

The experimental design was a strip plot with seven replicates (2.0 X 1.8 m plots). The horizontal strip was subsoil nitrate concentration [~0.3 m*M* NO_2-N (well water) or 20 m*M* NO_3-N], both of which were applied beginning 20 August 1994 through the subirrigation system at least weekly or after > 2.5 cm rainfall was received. The perpendicular strip was alfalfa germplasm, which included Agate, Ineffective Agate (a nodulated, but non-N_2-fixing near isoline of Agate), WL 322 HQ (a moderately dormant cultivar selected for high forage quality), Multi-7 (a selection with more than three leaflets per leaf), and four germplasms selected for varying root system architecture (low vs. high fibrousness in combination with tap vs. branch rootedness). These germplasms were seeded 12 June 1994, after inoculation with *Rhizobium meliloti.*

Alfalfa herbage was harvested on 19 August and 19 October 1994 and 16 June, 21 July, and 10 October 1995. The tracer, ^{15}N, was added in the nitrate form during five subirrigations from 22 June until 18 July at 0.4491 atom % ^{15}N. Herbage was analyzed for N and ^{15}N concentration by Dumas combustion followed by mass spectrometry. Nitrate uptake from the labeled subsoil nitrate supply and from symbiotic N_2 fixation was calculated using standard isotope techniques. Data were subjected to ANOVA.

Results and Discussion

Subsoil nitrate increased Ineffective Agate yield by 132%, but Ineffective Agate did not attain the yield of the N_2-fixing germplasms. Subsoil nitrate increased the yield of the N_2-fixing germplasms by 5% and there were marginal yield differences among the N_2-fixing germplasms ($P < 0.05$).

Subsoil nitrate increased total herbage N yield by 16%, but no differences among germplasms were found. Based on results from the summer regrowth period, where ^{15}N was applied, Ineffective Agate removed 41% more subsoil nitrate than the N_2-fixing germplasms. No differences in subsoil nitrate uptake were found among the N_2-fixing alfalfa germplasms and exposure to subsoil nitrate decreased N_2 fixation by 32% for each germplasm. However, we consider the results comparing effective germplasms to be too preliminary to draw firm conclusions at this time.

Summary

Results of this research confirm our earlier findings that ineffective alfalfa is superior to standard alfalfa for removing subsoil nitrate, despite typically lower yields with the ineffective line. Forage protein levels of ineffective alfalfa supplied with N often are nearly as high as in effective germplasms, making ineffective alfalfa a more valuable forage crop than a grass. As nitrate supply is depleted, the ineffective types will become chlorotic, thereby signaling when the site is remediated. Thus, ineffective alfalfa may be preferred for remediation of nitrate-contaminated sites. Commercial release of two ineffective alfalfas is scheduled for 1997.

Fine Root Turnover in Alfalfa During Stand Establishment

G.D. Goins and M.P. Russelle

Introduction

Fine root production and loss affect how ecosystems function because of plant nutrient and water uptake and subsequent release of assimilated C and nutrients to other organisms. Growth and decomposition in the fine root system occur simultaneously, and life spans of fine roots range from a few weeks to several years. Actual rates of fine root turnover (production, death, and decomposition) in the field are influenced by

nutrient and water availability, cultural practices (like harvest), pathogen infestation, and soil faunal predation.

Recently, alfalfa germplasms with different root system architectures have been produced via divergent phenotypic selection. These germplasms are being developed to serve different agronomic and environmental goals, such as rapid root elongation into the subsoil to help remediate nitrate-contaminated soil or high root length densities in the topsoil to absorb nutrients applied in agricultural and food processing wastes.

Knowledge of root demographic characteristics associated with these germplasms is important because rapid root turnover could help alleviate N deficiencies in eroded soils or in neighboring plants, but also might exacerbate a nitrate contamination problem in soils subject to nitrate leaching. Our objective was to determine the extent and patterns of fine-diameter root production and loss of four contrasting alfalfas during the stand establishment year.

Materials and Methods

An underground room was built in a loamy sand Udorthentic Haploboroll at the University of Minnesota Sand Plain Research Farm, Becker, MN. Minirhizotrons, plastic tubes 1.8 m long, 57-mm o.d., and 51-mm i.d., were placed horizontally and perpendicular to the walls at depths of 10, 20, and 40 cm from the soil surface, under different plant rows. Soil was fertilized and limed according to University of Minnesota recommendations.

Four alfalfa germplasms were seeded by hand (31 May through 2 June) directly over the tubes and border areas in 8 rows spaced 15 cm apart. Germplasms included: effectively nodulated Agate (AGATE); Ineffective Agate, an ineffectively nodulated non-N_2-fixing near isoline of Agate (INEFF); an effective germplasm having a tap root with few fibrous roots (LFTAP); and an effective germplasm with many fibrous roots and a strongly branch-rooted architecture (HFBRH). All were inoculated with *Rhizobium meliloti*.

Seedlings were thinned to 200 plants/m^2, irrigated according to a modified "checkbook" method, and received five biweekly fertilizer applications of 22 kg N/ha. Herbage was harvested on 19 Aug. and 20 Oct. 1994. Root samples were collected between adjacent plants within a row in early November by removing 32-cm diam. soil cores. Washed roots were stained and scanned to determine length.

Video images of roots were obtained biweekly. For each root in the 1920 images, date records were kept for root appearance, secondary thickening, and death. Three root cohorts were defined as roots produced in early-season (between 22 June and 20 July), mid-season (between 3 and 17 August), and late-season (between 31 August and 15 September). All fine roots produced within each interval and still alive at the end of the interval were included in a given cohort. Fine roots that became secondarily thickened at a later date were excluded from cohort analysis. Survivorship curves were generated and cumulative loss of fine root length was calculated from average turnover rates for the 0- to 30- cm and 30- to 60-cm increments measured with minirhizotron observations and from washed root lengths from soil cores.

Results and Discussion

Yields of the effectively nodulated germplasms were typical for this site, and INEFF yields were smaller, due to N limitations. Root length densities measured after washing soil cores showed no differences among germplasms nor interactions of germplasm with depth, but length densities declined from 13.7 km/m^2 in the upper 10 cm to about 3.3 km/m^2 at depths below 20 cm. Root length densities in the topsoil were significantly higher than those found in most other reports but were similar to those of commercial alfalfa in the Central Valley of California.

For all germplasms, net fine root production at the 10- and 20-cm depths rapidly increased for the first two months after planting, declined during mid-season, and then leveled off or declined slowly until autumn. By late October, differences in root

production among germplasms were evident at the 20- and 40-cm depths, but not at 10 cm. The HFBRH germplasm had 29% more fine roots than other germplasms at 20 cm. At the 40-cm depth, AGATE had fewer living fine roots than LFTAP and HFBRH, and both AGATE and INEFF had fewer total fine roots than HFBRH. LFTAP and HFBRH had similar numbers of fine roots at this depth, in contrast to 20 cm. About 7% of fine roots at each depth developed into secondarily thickened roots.

By the end of the first growing season, greatest fine root mortality had occurred at 10 cm (48%), and least occurred at 40 cm (36%). However, survival of contemporaneous root cohorts was not related to soil depth in a simple fashion. There was a pronounced loss (average 22%) of fine roots at the 10- and 20-cm depths in the 2-week period following herbage removal, confirming research in California, where fibrous root mass turnover was about 23% of that present before harvest in an established alfalfa crop.

Median fine root life spans at 10 and 20 cm ranged from 56 to 95 days for the early-season cohorts but only 42 to 69 days for the mid-season cohort. No estimates were possible for the 40-cm depth in the early-season cohorts or for any depths in the late-season cohorts. Lower herbage yield of the INEFF germplasm was not reflected in lower numbers of living or total fine roots produced by the end of the season. This confirms other results at this location, where total root mass, fine root mass, or fine root length was similar to ineffective and effective alfalfas, despite large differences in herbage yield.

Summary

Does root system architecture alter root demography? We did not find differences in total fine root turnover rates between the HFBRH and LFTAP germplasms, although our analysis of cohort survival demonstrated that a few temporal differences in root mortality rate occurred. Thus, although overall fine root survival during the stand establishment year was not influenced by root system architecture, the *patterns* of fine root mortality differed during the season. These mortality patterns will influence the timing of N release from decomposing roots. Based on a calculated loss of fine root length in the upper 60 cm of soil (totaling 274,000 km/ha) and an assumed N content of 0.22 mg N/m root, we estimate that fine root turnover in these alfalfa germplasms released about 60 kg N/ha during the stand establishment year.

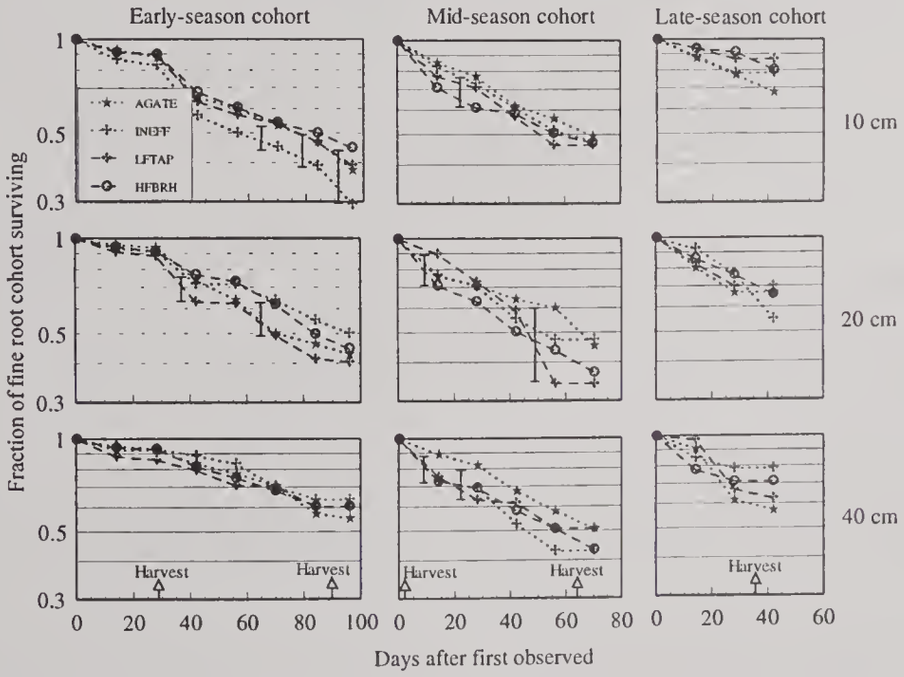

Figure 1. Fraction of surviving fine roots in three contemporaneous cohorts at three soil depths during alfalfa stand establishment. The period of observation extends to 26 Oct. 1994 in each case. For each date where significant differences were detected, the bar below the data points represents Fisher's protected LSD ($P < 0.05$) value.

Yield and N Uptake of Strip-Intercropped, Rotated Corn in a Ridge-Tillage System

T.K. Iragavarapu, G.W. Randall and M.P. Russelle

Introduction

Strip-intercropping, a practice in which two or more crops are grown simultaneously in contiguous narrow strips, is gaining popularity across the USA. In the Midwest, where continuous corn (*Zea mays* L.) and annual corn-soybean [*Glycine max* (L.) Merr.] rotations predominate, strip-intercropping with an additional small grain crop such as wheat (*Triticum aestivum* L.) provides diversity in both time and space and often improves overall system productivity. Strip-intercropping using a combination of reduced tillage and high levels of surface residue provided by the small grain also should reduce soil losses due to wind and water erosion compared to traditional practices. Ridge tillage may be well adapted to strip-intercropping because crops can be planted in the same rows year after year.

Planting legumes such as alfalfa (*Medicago sativa* L.) or hairy vetch (*Vicia villosa* Roth) either in or after small grains has increased yields and reduced the need for fertilizer N in the subsequent corn crop but is most successful where the autumn growing season is long. There are few reports of the fertilizer N requirement of corn when seeded after legume cover crops in reduced tillage systems in the Midwest and also little information on N requirements of corn in ridge tillage systems. Our objective was to provide such information on a soil common to much of the northern Corn Belt.

Materials and Methods

Field studies were conducted from 1991 through 1994 at Waseca and Freeborn in southern Minnesota on Webster clay loam soils (Typic Haplaquoll) on sites that produced soybean in 1990. Cropping sequences included continuous corn, soybean-corn, soybean-wheat-corn, soybean-wheat/hairy vetch-corn, and soybean-wheat/alfalfa-corn. Hairy vetch was seeded immediately after wheat harvest, whereas alfalfa was companion seeded with wheat. Legume seeds were inoculated with appropriate rhizobia. Sufficient plots were included in the four replicates so each phase of each rotation was present each year of the experiment. Strips of each crop were 4.6-m wide and 36.6-m long, except for continuous corn, which was grown in 9.2-m-wide strips.

Aboveground legume dry matter and N content were determined after the first killing frost each year. Every rotated corn plot was subdivided into four 9.1-m-long plots that received broadcast ammonium nitrate at 0, 45, 90, or 135 kg N/ha, and eight N rates were added to continuous corn plots (0 to 238 kg N/ha). Both grain and stover yields were determined after physiological maturity. Subsamples were analyzed for total N concentration. All data were subjected to either ANOVA (split plot arrangement in a randomized complete block design) or regression analyses.

Results and Discussion

Hairy vetch produced more aboveground dry matter and N than alfalfa in all cases except one site/year, but in no case was herbage N yield greater than 57 kg N/ha. These results are comparable to those for frost-seeded alfalfa into winter wheat in Michigan and for hairy vetch planted in a ridge-till system in Iowa in September, but smaller than N accumulations under milder autumn weather conditions.

Corn grain yield (Fig. 1) and aboveground N uptake (which paralleled yield) were lower in most treatments at the Freeborn site than at Waseca, probably because the former is imperfectly drained, whereas Waseca has parallel drainage tiles. Corn planting was delayed at Freeborn by several days in two of the years because of wet soil. At Freeborn, continuous corn grain yields and N uptake usually were smaller than corn grown after other crops; the soybean-corn rotation provided best yields in two of three years. In contrast, corn grain yields and N uptake generally were similar among rotations at Waseca, with the exception that they were lower following wheat alone in two of three years.

Differences among rotations at Freeborn cannot be explained by N availability because cropping systems generally had similar responses to added fertilizer N. Therefore, differences must be due to some non-N related rotation effects, such as autotoxicity from decomposing plant residues, that affected plant growth potential. Lack of response to the legumes at Freeborn contrasted with a positive effect at Waseca (relative to wheat alone), but the absence of larger responses was not surprising, given the small N accumulation in legume herbage. When legumes regrew after the mild winter of 1991-1992, they probably competed with corn for limited water and other resources, so corn yield was depressed by 1.5 Mg/ha following wheat plus alfalfa or hairy vetch at Freeborn. Effective control of interseeded legumes may require well timed use of herbicides under ridge-till conditions.

The similarity of corn response to applied N suggests that the cropping sequences did not differ in N use efficiency at fertilizer rates from 0 to 135 kg N/ha. However, it appears that higher rates would have been required to attain maximum yields in rotated corn, based on the substantial yield response to the final 45 kg N/ha increment. Continuous corn required 123, 207, and 199 kg N/ha to maximize grain yield at Waseca in the three years, which indicates that University of Minnesota recommendations for N (125 kg N/ha regardless of tillage regime) may be insufficient for ridge-till.

Summary

Based on these results, we do not recommend inclusion of alfalfa or hairy vetch with wheat as a consistent source of N for subsequent ridge-tilled corn. With excellent management and perhaps slightly higher fertilizer N rates, including wheat in strip-intercrops may produce corn yields that are competitive with those in the conventional soybean-corn rotation. Other benefits may also accrue, such as improved soil protection from erosion and lower pest activity due to increased crop diversity in strip-intercrops.

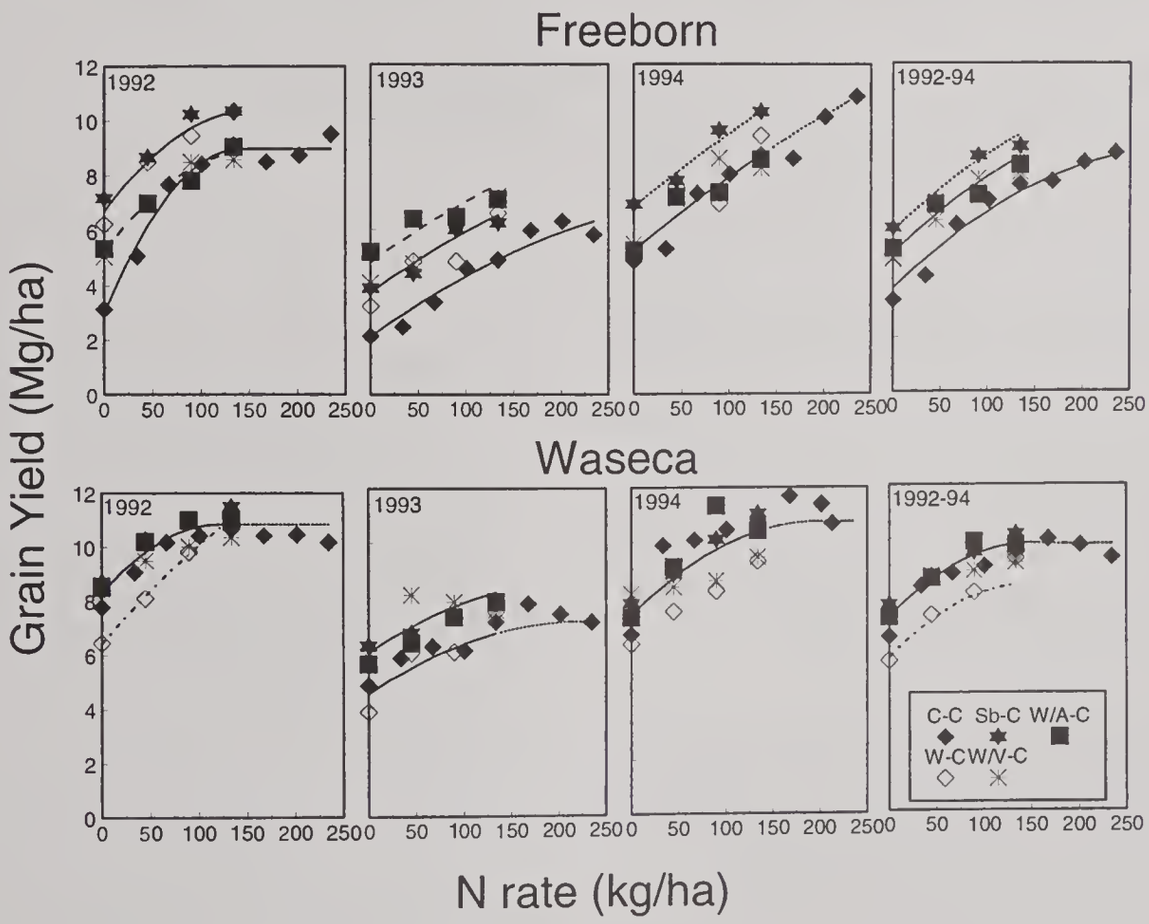

Figure 1. Grain yield (155 g/kg water content) of corn following corn (C-C), soybean (Sb-C), wheat alone (W-C), wheat/alfalfa (W/A-C), or wheat/hairy vetch (W/V-C) at Freeborn and Waseca, MN. Solid lines are for continuous corn and also when intercept and slope coefficients were identical for more than one cropping system.

Performance of Annual and Perennial Crops for Forage and Biomass Energy Production

I.C. Anderson, D.R. Buxton and A. Hallam

Introduction

An important aspect for economical forage production is high crop yield per unit of land. High yields allow fixed costs to be divided over more units of forage. In addition to their use for forage, the past decade has witnessed increased interest in forage crops for renewable energy sources. Thus, in addition to their use for forage, some crops could become important as dedicated energy crops. Seasonal yields of many warm-season, annual crops are reported to be greater than those of cool-season forage crops, but there have been few direct comparisons where the crops were grown under the same conditions. The purpose of this study was to determine yield and quality characteristics of several high yielding annual and perennial forage crops. The experiment was designed to evaluate the crops as potential energy crops, but the results have application to forage for livestock as well.

Materials and Methods

Reed canarygrass (harvested two times per year), switchgrass (harvested once per year), big bluestem (harvested once per year), sweet sorghum, forage sorghum (sorghum x sudangrass), and corn were grown near Ames, IA on highly productive flat soil and near Chariton, IA on marginal row-crop land with a 2-7% slope. The experiments contained four replicates at each site. These crops were fertilized with 0, 70, 140, or 280 kg N ha^{-1} (280 kg ha^{-1} is equivalent to 246 lbs N per acre). Also included in the experiments was alfalfa harvested two or three times per year. The crops were evaluated for fiber, nitrogen, and potassium concentrations.

Results and Discussion

The annual crops (sorghums and corn) produced about 50% more forage than the perennial crops at both locations (Figs. 1 and 2). The sorghums produced the highest average yields (over 16 t ha^{-1} or 7 tons per acre), especially at the lower nitrogen fertilizer rates. For most species, near maximal yields occurred at 140 kg N ha^{-1}. Corn yields at Ames were close to those of the sorghums at the

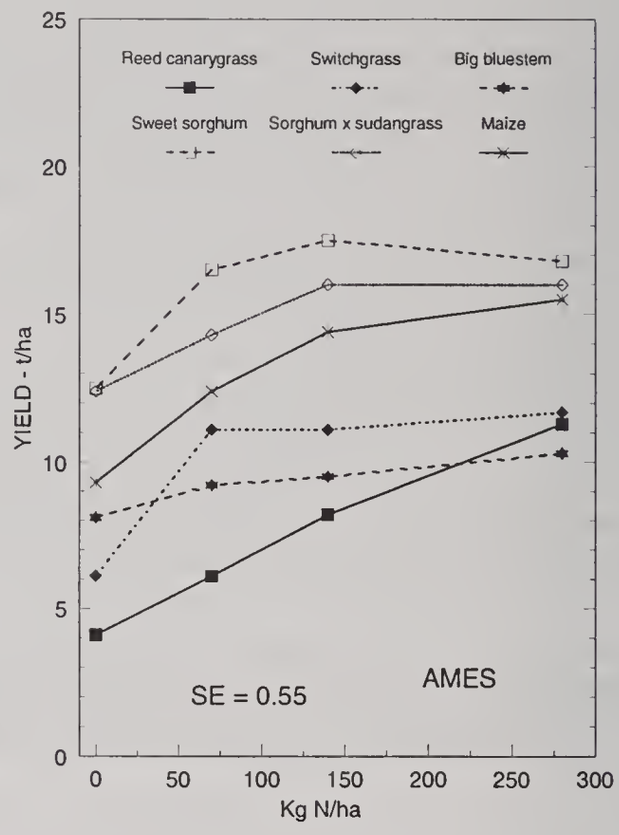

Figure 1. Yields of annual and perennial crops grown 1989 through 1992 near Ames, IA.

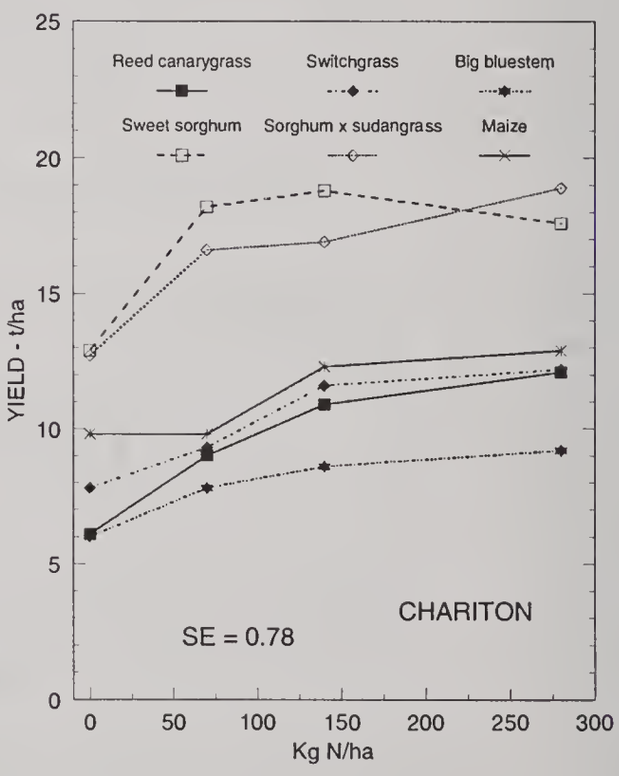

Figure 2. Yields of annual and perennial crops grown 1990 through 1992 near Chariton, IA.

maximal level of nitrogen. The less productive soils at Chariton had an adverse effect on corn production. Here corn often appeared water stressed and had yields that were only about 70% of the sorghums. Switchgrass generally out-yielded big bluestem. Reed canarygrass yield was near that of switchgrass only at the highest nitrogen level at Ames (Fig. 1) and similar to switchgrass at all nitrogen levels at Chariton (Fig. 2). Alfalfa produced 5 to 8% more forage with three cuts per year than with two cuts. Its yields were as high as 12 t ha^{-1}, but declined after 4 years of production.

Estimated annual soil erosion from the Universal Soil Loss Equation was less than 1 t ha^{-1} for all perennial grasses at both locations. That of alfalfa was less than 1 t ha^{-1} at Ames and less than 2 t ha^{-1} at Chariton. Conversely, estimated annual soil erosion for the sorghums and corn was about 5 t ha^{-1} at Ames and near 35 t ha^{-1} on the sloping soils at Chariton.

The chemical composition of the forage varied by species, but location and nitrogen fertilization generally had only minor effects. The major exception was that nitrogen fertilization increased nitrogen concentration of the forage. Highest neutral detergent fiber (NDF) concentrations occurred in big bluestem (80%), switchgrass (75%), and corn stover (72%), with intermediate values in reed canarygrass (65%). Lowest NDF values occurred in sweet sorghum (54%) and forage sorghum (48%). The NDF concentration was greater in 2-cut (56%) than in 3-cut alfalfa (49%).

Conclusions

Annual, warm season crops are more productive than traditional forage crops on both prime land and marginal land in the upper midwest. Although the sorghums were the most productive at both sites, the high potential for soil erosion on sloping soils would preclude their production on these soils. Here, switchgrass was among the highest yielding crops with production that generally increased during the course of the study.

DAFOSYM for Windows

C.A. Rotz and U.S. Gupta

Introduction

DAFOSYM is a simulation model of the dairy forage system. The model was developed as a research tool for evaluating and comparing alternative technologies for the dairy farm. The model was first used to evaluate feed production systems and later expanded to include manure handling and tillage systems. By simulating different systems for the same base farm, performance and economic results can be compared to determine the best system. DAFOSYM also provides an excellent teaching aid for use in classrooms and Extension workshops. The model illustrates the complexity of the many interactions among components of the dairy farm. For the experienced user, the model may also provide information useful for strategic planning of dairy farms. Work was undertaken to convert DAFOSYM to a Windows operating system to improve its usefulness to others and to enable further expansion of the model.

Methods

DAFOSYM is a simulation model of crop production and feed use on dairy farms and the return of manure nutrients back to the land. The dairy forage system is simulated over many years of weather to determine long-term performance and economics of alternative technologies and/or management strategies. By modeling several alternatives on the same representative farms, those alternatives which maximize farm production or profit can be determined.

The model is adapted to different locations by changing weather and soil input parameters. The alfalfa growth routine predicts dry matter

accumulation and quality changes on a daily basis throughout the growing season. When the crop is ready for harvest, the harvest routine simulates field machinery operations, drying, and rewetting in 3 h increments. Losses and quality changes due to machine operations, plant respiration, and rain damage are accounted to predict the quantity and quality of forage stored. A corn model predicts corn grain and silage yields, and a harvest routine accounts for losses and resource requirements during harvest. Storage losses and associated quality changes are predicted for dry hay, silage and grain stored by different methods. Following storage, feeds are either sold or allocated to a dairy herd. For the dairy herd, balanced diets are fed to each of six animal groups with higher quality forage fed to high producing animals. Supplemental feeds are purchased as needed and extra feeds are sold.

Manure production is modeled as feed dry matter (DM) consumed minus the digestible DM extracted by the animals plus urine DM and any feed DM lost into the manure. The quantity of manure handled is influenced by the type and amount of bedding used and the manure handling method. Nutrients in the fresh manure are determined through a mass balance of the six animal groups. Manure nutrients equal the nutrient intake minus nutrients contained in milk produced and in meat produced through animal growth. Nutrient losses are subtracted to determine that available for plant growth. Crop nutrient requirements are based on the nutrients removed by field crops as a function of yield. These requirements are met with purchased fertilizer minus credits from crop rotation carryover and manure.

Moisture near the soil surface is tracked through time to predict days suitable for field work. Soil moisture is increased by rainfall and decreased through evapotranspiration and moisture flow to lower soil layers. Field operations are allowed only on days when the moisture is below a critical level. Up to six sequential operations are used for establishment of each crop. On any given parcel of land, the operations must occur in a sequence. Tillage follows manure handling in the sequence of spring and fall operations. A delay in planting due to untimely operations results in a decrease in corn yield.

An economic analysis includes all costs associated with growing, harvesting, storing and feeding of crops to the milking herd and young stock and the collection, storage, and application of manure back to the crop land. Total feed and manure costs are determined as the sum of all costs associated with these processes. Additional costs for animal housing, milking, herd health, and herd maintenance are then included to estimate the total production costs and the net return to management or farm profit. All production costs and the net return over these costs are determined for each simulated year of weather conditions. The distribution of annual values obtained can be used to assess the risk involved in alternative technologies or strategies as weather conditions vary.

Results and Discussion

DAFOSYM for Windows is primarily intended for use as a teaching tool. This tool can be used in university courses in Bio-Systems Engineering, Agronomy, and Dairy Science. Students can use DAFOSYM to learn more about the complexity of interactions that occur within a dairy production system. The model can also be used in Extension type workshops. With some training, Extension field staff, private consultants, and producers can use the model to study the impacts of various technology changes on farms in their area.

The user interface functions like other well designed Windows based programs. Icons are used to direct the user through major model functions. Menus are used to view or modify model parameters. Files are supplied with the model that provides default values for all parameters of example farms. Parameters are changed by reentering values in an entry box, selecting the appropriate option from a list box, or setting the desired value through a scroll box. Either metric or English units of measurement can be used.

DAFOSYM for Windows functions on any computer that uses Microsoft Windows version 3.1 or later, but it works best on computers using a 486 DX or Pentium processor with at least 4 MB of RAM. About 5 MB of disk space is required to store the program and its associated data files. A Windows type help system assists the user in

preparing a simulation and interpreting the results. DAFOSYM is published and distributed by the USDA's Agricultural Research Service. The model and associated files are available without charge through the World Wide Web from the home page of the U.S. Dairy Forage Research Center (http://www.dfrc.wisc.edu).

Conclusion

DAFOSYM for Windows is available through the home page of the Dairy Forage Research Center. It is provided as a teaching aid that illustrates the complexity and interaction of the many components of the dairy farm.

Economics of Grazing Alfalfa on Michigan Dairy Farms

C.A. Rotz

Introduction

Costs of feed production and manure handling are increasing more rapidly than the price of milk, placing an economic squeeze on dairy farmers. Decreasing profit is causing many to look for ways to reduce their costs. One option is the use of rotational grazing systems to reduce feed costs. A deterrent to the adoption of grazing is the lack of good information on the long-term economic benefits of grazing. Although many farmer testimonials are heard, well documented comparisons of grazing and confined feeding systems are seldom found. Such comparisons are difficult because variations in weather across locations and years obscure the data that must be compared. A proper comparison must be made over many years of weather accounting for equipment, material and labor requirements, forage losses, and feed supplementation. A model of the dairy forage system (DAFOSYM) provides a tool for performing such an analysis.

Methods

To evaluate the costs and benefits of grazing in Michigan, DAFOSYM was used to compare the long-term performance and economics of confined feeding and grazing systems on a representative dairy farm. Farm simulations showed how grazing of alfalfa affects feed requirements, manure handling, overall feed and manure costs, and the risk or year-to-year variation of these costs. A further analysis was conducted to determine the sensitivity of the predicted results to assumptions for herd production level, crop productivity under grazing, machinery life, and grazing management costs.

The farm included 100 milking animals plus replacement stock on 250 acres of owned land. Essentially all forage and grain feeds required by the herd were produced on the farm. The same machinery and structures were used for both confined feeding and grazing systems. Grazing systems required additional investments in fence and watering equipment. Fence included both high tensile perimeter fence and electric fence used to form paddocks. Labor for grazing management was assumed to be 5 h per week during the grazing season. Milk production levels of 18,000 and 20,000 lb/cow were used. Simulations were done for 25 weather years using East Lansing, Michigan weather.

Results and Discussion

Use of rotational grazing along with good feeding management provided a substantial reduction in the use of conserved forage, corn, and soybean meal on this representative farm. The simulated feeding strategy replaced TMR with grazed alfalfa based upon availability. The TMR was balanced to meet the energy and protein needs of the animals while considering the quantity and quality of grazed alfalfa consumed. The net result was the use of about 40% less alfalfa hay and silage, 35% less corn silage, 10% less corn grain, and 25% less soybean meal.

Use of grazing provided an economic advantage over confined feeding. Equipment and material costs were similar between the systems because the amortized cost of fence and watering equipment was largely offset by the cost saving obtained through less hours of use of harvesting, feeding, and manure

handling machinery. This reduction in machinery use also reduced fuel and electric use about 33%. With less machinery use, less labor was required. The labor saving was partially offset by grazing management labor giving a net reduction of 26%. Seed, fertilizer, and chemical costs were reduced 23% with grazing primarily because less corn was produced. About 34% less bedding was required with 34% less manure hauled each year. Altogether, these effects provided a 12% reduction in the average feed and manure handling cost. Grazing the 18,000 lb herd reduced these costs by $0.83/cwt of milk produced compared to the confined feeding system. At a production level of 20,000 lb/cow, the cost reduction was slightly less at $0.73/cwt. The net return or profit margin of the farm was increased by $146/cow or $58/acre.

The use of grazing did increase the risk in maintaining feed costs. The variation in feed and manure handling costs for the grazing system over many years of weather was 40% greater than the variation of those costs with confined feeding. This occurred because the annual fluctuation in yield for grazed alfalfa due to the influence of weather was higher than the variation of the average production of all harvested feeds. Even though the variation was greater with grazing, feed and manure costs were always less with grazing.

A major assumption in this analysis was that the same milk production was maintained for both the confined feeding and grazing systems. Further analysis determined that the dairy producer could accept up to a 1,600 lb/cow decrease in milk production with this grazing system in Michigan and still obtain a greater profit than the alternative with confined feeding. The sensitivity of several other assumptions of the analysis are noted in Table 1.

Conclusion

Grazing of alfalfa is an economically viable option for dairy farms in Michigan. The grazing strategy used and other assumptions of the analysis affect the benefit received. With the strategy evaluated in this study, many of the inputs in feed production are reduced and the need for purchased feeds is reduced. The overall result is an increase in the annual return to management or farm profitability of $100 to $240/cow.

Table 1. The reduction in cost and the increase in net return attained through grazing of a herd producing 20,000 lb/cow and the effect of changes in certain assumptions used to describe the grazing system on this cost and net return.

Change in grazing system	Reduction in feed & manure cost ($/cwt)	Increase in net return ($/cow)
Base grazing system	.73	142
20% lower yield of grazed alfalfa	.50	98
6 year alfalfa stand life	.77	150
20% greater fence costs	.68	132
10 h/wk for grazing management labor	.66	128
14 year machinery life	1.05	238
Smaller equipment and forage structures	1.08	212
40% culling rate and bloat control additive	.71	111

Simulation of Dairy Manure Management and Cropping Systems

T.M. Harrigan, W.G. Bickert and C.A. Rotz

Introduction

In recent years, environmental concerns regarding surface and groundwater quality have led to an increasingly restrictive, tightly regulated and costly operating environment for livestock producers. Legislation has been enacted at the Federal level to regulate tillage and manure management practices. Most states have Right-to-Farm legislation to protect crop and livestock producers from nuisance suits if they follow recommended or best management practices. The challenge for dairy farmers is to select and manage tillage and manure handling systems in a cost effective and environmentally safe manner. An analysis of the complex interactions of tillage and manure management with other operations and processes on the dairy farm requires a systems approach. This analysis must integrate the effects of weather, machinery, labor, tillage, planting, manure handling and other relevant factors on the management of the dairy herd. DAFOSYM, a simulation model of the dairy forage system, provides a basis for such an analysis.

Methods

DAFOSYM is a comprehensive computer model that simulates alfalfa and corn growth, harvest, storage, feeding, and manure production on a dairy farm. Submodels were added to enable an evaluation of the interaction of manure storage and application with tillage and planting. New submodels were developed to predict suitable days for tillage, planting and manure application operations under a range of soil and crop residue conditions; draft of a wide range of tillage and seeding implements; and scheduling of tillage, planting and manure application operations.

The timeliness of tillage, planting and manure handling is influenced by labor availability and the type of equipment used. Ten hours were available for field work during each suitable day. Manure spreading began in the spring as soon as the soil was thawed and trafficable. Manure application was immediately followed by the sequence of tillage and planting operations. Fall tillage and manure spreading began after corn silage was harvested and continued as land became available following corn grain harvest. Corn grain harvest was custom hired and thus did not interfere with the scheduling of tillage or manure spreading operations. A fourth cutting of alfalfa began in mid-October and this could delay tillage and manure hauling. Depending upon equipment and labor available, tillage and manure handling were scheduled either in series, where completion of manure spreading was required before tillage could begin, or as parallel operations where tillage and spreading progressed simultaneously.

Three tillage systems were modeled. Conventional tillage included fall tillage with a moldboard plow. In the spring, corn land was disked once and field cultivated once before planting. Alfalfa land was disked twice and field cultivated twice before seeding. Mulch-tillage included primary tillage with a coulter-chisel plow in the fall and spring seedbed tillage with a combination disk/field cultivator/coil-tine harrow. Land to be planted in corn required one pass for manure incorporation and seedbed preparation in the spring while alfalfa land was worked twice prior to seeding. A modified no-till system included fall tillage with a rolling-tine aerator. The aerator buried very little residue yet loosened the soil to improve water infiltration and alleviate shallow soil compaction. Crops were planted with a zone-till planter.

Manure handling systems included short-term storage with frequent hauling in a V-tank spreader and three systems using long-term storage: slurry tanker spreading, slurry tanker injection, and slurry irrigation. Manure was removed from long-term storage by irrigation or hauling with top loaded slurry tankers. Seven months storage capacity was provided. Pit agitation and tanker loading used a tractor powered pump/agitator. Agitation began two hours prior to the start of spreading and then continued only during tanker loading. Agitation was continuous during irrigation. The average hauling distance for manure was 0.8 km for the 150-cow

herd and 2 km for the 400-cow herd. A pressure pump capable of pumping up to 1 km was located at the storage pit when manure was irrigated. An auxiliary pump was added to extend the pumping distance for the 400-cow herd. Slurry injection was not used with the modified no-till system.

Results and Discussion

The highest machinery, fuel and labor costs for manure hauling were associated with slurry injection and the lowest with daily hauling. Costs for daily hauling were sensitive to spreader loading method. Fuel and labor costs were similar to slurry systems when the spreader was loaded with a front-end loader. Direct loading of the spreader from a push-off ramp reduced fuel use in handling by 20 to 40% and labor use by 60 to 80% compared to bucket loading. The manure system used had a large effect on the timeliness of tillage and planting. Daily hauling of manure distributed labor throughout the year and allowed little interference with the timeliness of tillage and planting. Long-term manure storage concentrated labor for spreading in the spring and fall. Injection or surface spreading of stored slurry delayed tillage and planting and increased feed costs as much as $24/cow-yr when manure hauling, tillage and planting occurred in series. When labor and machinery were available for parallel field operations, manure handling method had little effect on the timeliness of tillage and planting.

The highest machinery, fuel and labor costs for tillage and planting were associated with conventional seedbed tillage. Compared to conventional tillage, mulch-tillage reduced machinery, fuel and labor costs about 30%; the modified no-till system reduced machinery costs about 25%, fuel costs 45% and labor costs, 50%. The tillage system used had a large effect on the timeliness of fall tillage. Chisel plowing was finished about two weeks earlier and soil aeration about four weeks earlier than moldboard plowing. There was little difference in timeliness of planting between tillage systems.

Compared to slurry injection, daily hauling increased net return as much as $54/cow-yr on the 400-cow farm and $78/cow-yr on the 150-cow farm. This economic advantage for daily hauling diminished if credit was not given for the fertilizer value of the manure nutrients when spread daily. Manure irrigation increased net return $23 to $29/cow-yr over slurry injection. The highest net return among tillage systems was associated with mulch-tillage, returning $15 to $25/cow-yr over conventional tillage. Use of the modified no-till system increased net return $6 to $16/cow-yr over conventional tillage, but when compared to mulch-tillage, savings in fuel and labor were more than offset by higher costs for seed, fertilizer and pesticides.

Conclusion

The expanded DAFOSYM model provides a flexible and useful tool for comparing the long-term performance and economics of tillage and manure handling systems and their interaction with feed production on dairy farms.

Alfalfa and Corn Silage Systems Compared on Michigan Dairy Farms

L.R. Borton, C.A. Rotz, J.R. Black, M.S. Allen and J.W. Lloyd

Introduction

Primary forages for Michigan dairy herds are corn silage and alfalfa. Feeding trials generally demonstrate similar milk production from corn silage and alfalfa based diets when the forages are properly balanced and fed. Given that similar milk production can be attained, overall farm performance and economics become key issues in forage selection. Past studies do not conclude that one forage is always better than the other using economic criteria. A new look at this comparison is needed due to recent changes in the dairy industry. These changes include higher herd lactation averages and the increasing importance of nutrient management. Also, computer technology can now provide better analyses through simulations that integrate weather risk and the many interactions among farm components. A study was conducted to

determine the best combinations of alfalfa and corn forage for representative Michigan dairy farms considering machinery and labor utilization, nutrient loss to the environment, and overall farm profitability.

Methods

DAFOSYM was used to compare the relative merits of forage systems when 0, 1/3, 2/3 and all of the forage requirements on a dairy farm came from corn silage with the remainder from alfalfa. Primary comparisons were the net return above feed and manure costs, but manure management issues and labor requirements were also considered. DAFOSYM simulates the growth, harvest, storage, and use of alfalfa and corn along with manure production, collection, storage, and application to crop land on representative dairy farms. Simulation over many years provides a distribution of annual values of farm performance, costs and economic returns as influenced by weather. This study required the simulation of representative farms synthesized through expert opinion and surveys and a sensitivity analysis to determine the impact of major assumptions.

The primary farm studied included 120 lactating and dry cows, but farms of 60, 250 and 400 cows were also used to determine effects of farm size. Milk production goals were selected to represent above average (8,000 kg/cow/yr) and very high (10,500 kg/cow/yr) production levels. The farms were simulated for 26 years of historical weather for East Lansing, Michigan. Soil types chosen were representative of clay loams and sandy loams in Michigan. Silo and machinery sizes were selected to maximize farm net return under each cropping strategy. Manure handling, storing, and application were represented by three systems: solid, spread slurry, and injected slurry. Slurry was chosen as the primary manure system for this analysis to make best use of manure nutrients.

A partial budgeting analysis was used to compare forage systems. Major factors included in the analysis were the labor, machinery, supplies, and energy associated with growing, harvesting, storing and feeding crops as well as handling and applying manure. Long term relative prices were used for feed and milk to mitigate the impact of fluctuating prices. Historical prices were used to establish the long-term price ratios of hay, soybean oil meal, and milk relative to corn.

Results and Discussion

A comprehensive comparison of forage systems representing a range from all alfalfa to all corn silage tended to show the highest net return over feed and manure costs with all alfalfa systems. Relative differences in net returns among the ratios of corn silage and alfalfa studied were small compared to the year-to-year variation in net return caused by weather. These differences are not large enough to encourage a sudden reallocation or reinvestment of resources.

Farm size did not have much effect on the comparison of the four forage systems. Systems using 2/3 or all corn silage had the lowest net returns over feed and manure costs (Table 1). The all alfalfa system remained the most economical, but differences across systems were small. The standard deviation or variance in the net returns across years of weather decreased as farm size increased. Reasonable changes in soil type, milk production level, relative prices, and other major assumptions used in the analysis had small effects on the differences in net return across systems and the relative ranking of systems. These changes sometimes greatly reduced the differences in net return across systems, and never provided a substantial increase in these differences.

For best use of labor and manure resources on the farm, a forage ratio of 1/3 to 2/3 corn silage was needed. Use of more than one forage crop spread labor requirements more uniformly throughout the cropping season. An all alfalfa system required most of the manure to be applied to alfalfa; a practice that is normally discouraged to promote weed control, stand persistence, and thus maximum yield. All alfalfa also produced large amounts of excess nitrogen. If this nitrogen does not reduce nitrogen fixation by the alfalfa crop, ground water contamination may be an environmental concern. Given the lack of a strong economic advantage

among the forage systems, the practice of having 1/3 to 2/3 of the forage requirement provided by corn silage is favored to improve manure and labor management.

Conclusion

Although all alfalfa forage systems may provide a slight economic advantage, labor and manure nutrient utilization issues confirm that between 1/3 and 2/3 of the forage requirement on Michigan dairy farms should come from corn silage.

Table 1. Net returns per cow over feed and manure costs for four farm sizes and four portions of corn silage and alfalfa.*

Corn silage (CS) portion of total forage								
Farm	No CS		1/3 CS		2/3 CS		All CS	
size	mean	CV†	mean	CV	mean	CV	mean	CV
60 cow	$1,574	7.6%	$1,544	7.6%	$1,487	8.7%	$1,519	8.6%
120 cow	1,727	5.8	1,665	6.2	1,646	7.4	1,658	7.8
250 cow	1,817	5.2	1,783	5.4	1,769	6.4	1,755	7.2
400 cow	1,846	5.1	1,816	5.2	1,794	6.3	1,784	7.1

*Numbers represent differences between means averaged over 26 years of historical weather.
†CV is coefficient of variation or 100 times the standard deviation divided by the mean.

A Simulation Model of Cold Hardiness and Freezing Injury in Alfalfa as a Function of Cultivar Type

V.R. Kanneganti, R.P. Walgenbach and C.A. Rotz

Introduction

Alfalfa yield, persistence and profitability are affected adversely by winter injury in colder climates of North America. The extent of crop injury varies widely, causing large year-to-year fluctuations in yield and associated profitability. In years of adverse winter weather, production losses may amount to millions of dollars. By reducing yield and stand life, winterkill affects N fixation and soil-N uptake, thus influencing farm N budgeting and the environment. For these reasons, effects of winter injury cannot be ignored in alfalfa models, particularly when these models are to be used in whole farm simulators such as DAFOSYM for evaluating alternative management options in relation to production, profitability, or the environment.

Existing models of alfalfa lack winter injury effects or do not differentiate cultivar types for their differential response to winter survival and yield during multiple years of an alfalfa crop. ALSIM1, the alfalfa model used in DAFOSYM, does not simulate over-winter processes such as cold hardiness, freezing injury or stand loss. Consequently, output analyses of DAFOSYM lack winter-kill effects.

The objectives of this work were: (1) Develop a process-based module of cold hardiness and freezing injury for alfalfa. (2) Link the module to existing alfalfa growth models. (3) Validate the combined model (growth model + cold hardiness module) for predicting the effects of freezing injury on yield and stand life as a function of cultivar type and cutting management during 2-4 years of continuous crop growth.

Model Development

Model structure and components. Cold hardiness and winter injury are newly developed. Other components in the model are adapted from existing

models (Fick, 1981; Denison and Loomis, 1989). The state variables of the model include leaf blade, stem (includes flowers and seeds), buds, crown, root, and carbohydrate reserves. The processes include photosynthesis, shoot and root growth, dynamics of storage reserves, cold hardiness, freezing injury, and evapo-transpiration. The model simulates crop and soil processes daily.

Cold hardiness and freezing injury. Even though the mechanisms of dormancy and winter hardiness are not fully understood, studies have shown a strong correlation of cultivar dormancy characteristics to cold hardiness and winter survival (Cunningham et al., 1995; Jung et al., 1967; McCaslin, 1994; McKenzie et al., 1988; Schwab, 1993; Smith et al., 1986). These studies concluded that cultivar types of all dormancy ratings tolerated lower freezing temperatures as cold hardiness increased. However, cold-sensitive cultivars suffered higher rate of plant death at similar freezing temperatures compared to the winter-hardy cultivars. Based on these data, accumulation of cold hardiness was developed as a function of cultivar type which in the model is characterized by fall dormancy rating (Fig. 1). Fall dormancy ratings (FDR) are supplied as user input. Cultivar ratings for dormancy are routinely published by the seed companies or are available from cultivar evaluation trials. Besides cultivar type, the rate of accumulation or break-up of cold hardiness is further modified by temperature and snow cover. Carbohydrate reserves affect cold hardiness accumulation only when the reserves in the root and crown fall below 10%. The process of winter acclimation resulting in cold tolerance is modeled with a simulated cultivar hardiness index. Cold tolerance to freezing injury increases as the hardiness index increases.

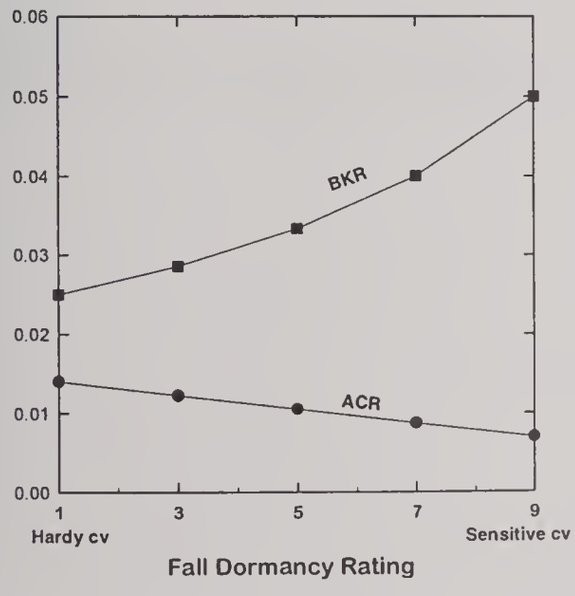

Figure 1. Potential rate of accumulation (ACR) or break-up (BKR) of cold hardiness for different cultivar types plotted as a function of fall dormancy rating (FDR). (Data derived from studies on accumulation of soluble sugars and protein in different cultivar types in response to freezing temperature.)

Model inputs. Model requirements for user input data include: (a) daily weather (temperature, precipitation, and solar radiation), (b) soil (water holding capacity by layers), and (c) cultivar rating for fall dormancy.

Model Validation

Forage Yield Prediction. Field measured yield data were obtained from published sources (Djajanegara, 1990; Lang, 1985; Tesar, 1984) to test model predictions of forage yield. The validation data consisted of a total of 82 yield data representing different combinations of cultivars, production years, and cutting management systems at three locations across the north-central U.S. during 1977-90. Cutting schedules included 3, 4 or 5 harvests per yr. During the winters of 1988-89 and 1989-90, significant yield loss due to winterkill was observed in WI. Dormancy ratings for the cultivars tested varied between 2.5 and 4.0. Model predictions of yield were simulated for the corresponding field measured data by running the model for 2 to 4 years continuously.

The need for cold hardiness and winter injury simulation for predicting yield was tested by comparing model predictions of forage yield with or without winter injury components to the corresponding field data (Table 1). Values of (model-field) greater than zero represent over-predicted yields, while values less than zero represent under-predicted yields. Without winter injury simulation, yield was over-predicted by 0.95 Mg ha^{-1} cut^{-1} or 2.94 Mg ha^{-1} yr^{-1} compared to the corresponding field data (MOD_{NO}-FLD, all years, Table 1). During years of winterkill, over-prediction was greater (1.30 Mg ha^{-1} cut^{-1} or 5.83 Mg ha^{-1} yr^{-1}, 1988-90, Table 1), resulting in prediction errors of up to 50%.

Simulation of cultivar hardiness to winter injury improved yield prediction significantly (MOD_{NO}-MOD_{YES}, Table 1). Model predicted yields were within 0.42 Mg ha^{-1} (14%) for individual harvests or 1.15 Mg ha^{-1} (8%) for annual yield compared to the field data (MOD_{YES}-FLD, all years, Table 1). During years of winterkill, prediction errors were within 8% (MOD_{YES}-FLD, 1988-90, 0.22 Mg ha^{-1} cut^{-1} or 0.99 Mg ha^{-1} yr^{-1}, Table 1).

Conclusion

(1) Simulation of cold hardiness and winter injury as a function of cultivar type improved forage yield prediction significantly. The model is capable of predicting yield in colder climates for different cultivar types managed under a variety of cutting schedules for 2 to 4 years continuously. (2) While this model was developed for use in DAFOSYM, other potential applications of the model include: (a) As a prediction tool to forecast winterkill each year. (b) As a tool in developing "cultivar maps" of winter injury for different cultivar types as a function of weather and cutting management.

Model availability. The model is written in FORTRAN 77. The computer code and documentation are available upon request.

Acknowledgments. The authors wish to thank D.K. Barnes, M.P. Russelle and M.B. Tesar for their assistance.

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Table 1. Comparison of model predicted forage yield with the corresponding field measured data for a single harvest or for annual production.

Abbreviation and description	All years				Winterkill years (1988-90)			
	Single harvest (n† = 62)		Annual yield (n = 20)		Single harvest (n = 18)		Annual yield (n = 4)	
	mean	s.d.‡	mean	s.d.	mean	s.d.	mean	s.d.
	— Mg ha^{-1} —		— Mg ha^{-1} —		— Mg ha^{-1} —		— Mg ha^{-1} —	
FLD: field measured data	2.90	1.39	13.63	3.83	2.60	1.33	11.68	1.85
MOD_{NO}: model without cold hardiness and winter injury	3.85	1.42	16.57	3.31	3.89	1.45	17.51	3.44
MOD_{YES}: model with cold hardiness and winter injury	3.32	1.37	14.77	3.08	2.81	1.44	12.66	1.41
MOD_{NO}-FLD: MOD_{NO} compared to field data	0.95	0.99	2.94	3.26	1.30	0.89	5.83	2.99
MOD_{YES}-FLD: MOD_{YES} compared to field data	0.42	0.80	1.15	1.80	0.22	0.82	0.99	1.20
MOD_{NO}-MOD_{YES}: Without or with winter injury	0.53§	0.69	1.80¶	2.48	1.08§	0.83	4.85#	3.86

Intensive Mechanical Forage Conditioning: Relationship to Increased Animal Utilization

T.J. Kraus, R.G. Koegel and R.J. Straub

Introduction

Intensive conditioning of forage has been shown to improve animal utilization, especially the amount of energy derived from the fiber fraction (Koegel et al. 1992). This raises questions as to the appropriate level of conditioning to realize the potential benefits without over-conditioning, since this increases both capital and operating costs needlessly.

Establishing a relationship between degree of conditioning and increase in animal utilization requires: (1) an objective quantitative measurement of the degree of conditioning and (2) studies which relate rate and/or extent of forage disappearance (in vitro, in situ or in vivo) to degree of conditioning. Such a study was undertaken.

Methods

Forage samples were prepared and placed in polyester bags to determine in situ disappearance in the rumen. Materials and treatments were: 2 plant species x 3 maturities x 4 conditioning levels x 4 digestion times x 3 fistulated cows (for each species) x 3 replicates = 864 samples.

The materials and treatments used in the in situ study were as follows:
Plant species: alfalfa (3rd cutting) and orchard grass (3rd cutting)
Maturities: 1. late vegetative/prebud, 2. late bud/ early flower, 3. late flower/early seed
Conditioning: 1. control, 2. moderate crushing-impact, 3. severe crushing-impact, 4. rotary-impact maceration
Digestion Times: 6, 12, 24, 48 hours

Level of conditioning was measured as the conductivity of forage leachate prepared in a prescribed manner (see USDFRC 1994 Research Summaries).

The rationale for the conductivity measurement was that the higher the number of disrupted cells, the greater the concentration of electrolytes in the leachate. In addition to measuring the leachate conductivity of each species-maturity-conditioning level combination, each of the six species-maturity combinations was treated in a Waring blender and the conductivity of the resulting leachate measured. This was considered the "ultimate" treatment which would give the maximum attainable leachate conductivity and this would be dependent almost entirely on herbage chemistry. The three conditioning levels were chosen to give conductivities which were well distributed between the control and Waring blender values. Relative conductivity (RC) was defined as the conductivity ratio (%) of a conditioned forage to that of the same forage treated in the Waring blender.

Results

Conductivity values for the three alfalfa controls averaged 51 micro-siemens while corresponding values for the most severe treatment level averaged 1067. Average conductivity for the Waring blender treatments was 1380 giving average relative conductivities (RC) of approximately 3.7% for the controls and 77% for the most severe treatment.

Dry matter disappearance from the polyester bags is plotted vs. time in Figures 1 and 2 for the least (1) and most (3) mature alfalfa. These data are fitted with straight lines on a log-log plot in accordance with the usual model: disappearance = $A\ (1\text{-}e^{-kt})$. R^2 values for all fits in Figs. 1-3 exceed 0.95. It can be seen at 6 hr and 12 hr that the % disappearance increases with the severity of the treatment or RC value. By 48 hours the data points for the different treatments tend to converge. The increase in 6 hour DM disappearance of the most severe treatment relative to the untreated control is 28% for the least mature alfalfa and 40% for the most mature.

Figure 3 is a plot of 6 hr dry matter disappearance vs. relative conductivity (RC), a measure of conditioning severity, for the three levels of maturity. Here dry matter disappearance increases with conditioning severity (RC). The largest benefit in dry matter disappearance occurs with the first increment of conditioning severity. Successive increments of conditioning severity result in ever smaller increases in DM disappearance. While the slopes of the lines fitted to the data become smaller at higher levels of conditioning, there appear to be small increases in disappearance even at the highest level of conditioning (70.9 RC $\leq$ 86.8) used in this study.

Conclusions

Electrical conductivity of forage leachate appears to be a useful and convenient measure of level of mechanical conditioning. In addition to correlating well with the severity of treatment, it also correlated well with the rate of disappearance of forage dry matter from polyester bags placed in the rumen. These observations agree with earlier in vitro studies (Koegel et al, 1992) showing higher digestibility of severely conditioned forage than of control forage.

Reference

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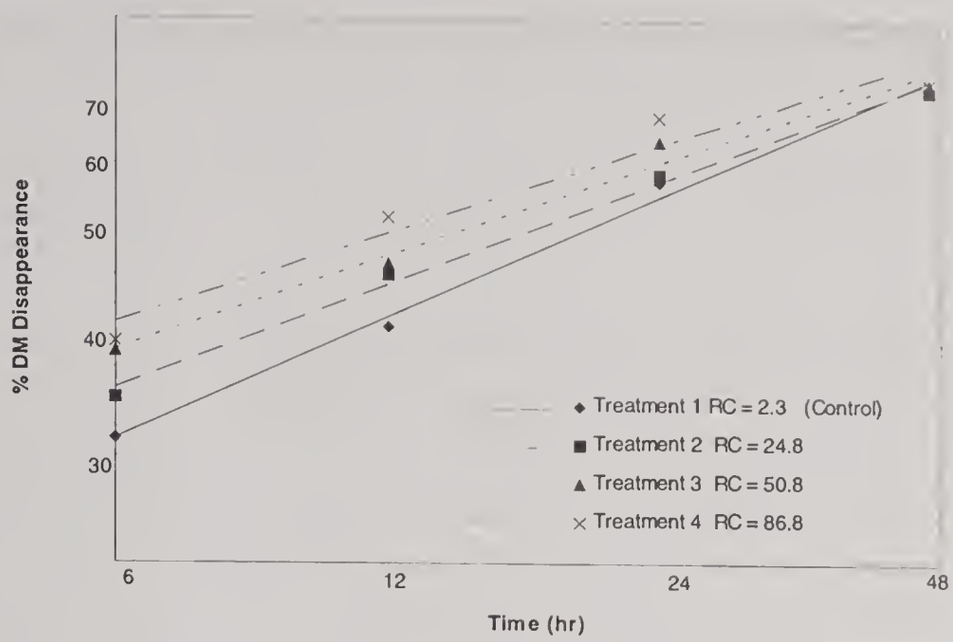


Figure 1. % DM disappearance vs. time. Alfalfa - maturity 1.

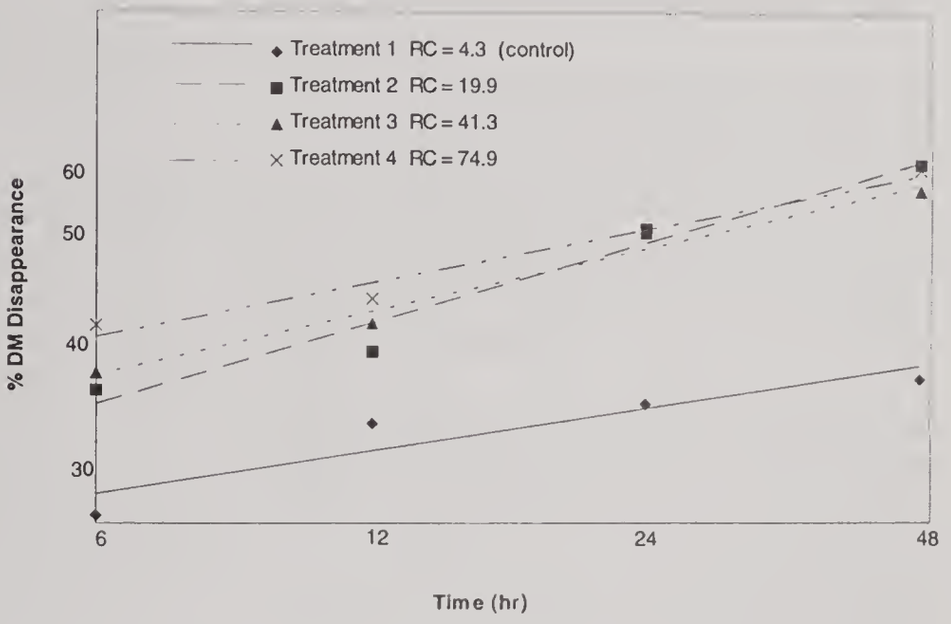


Figure 2. % DM disappearance vs. time. Alfalfa - maturity 3.

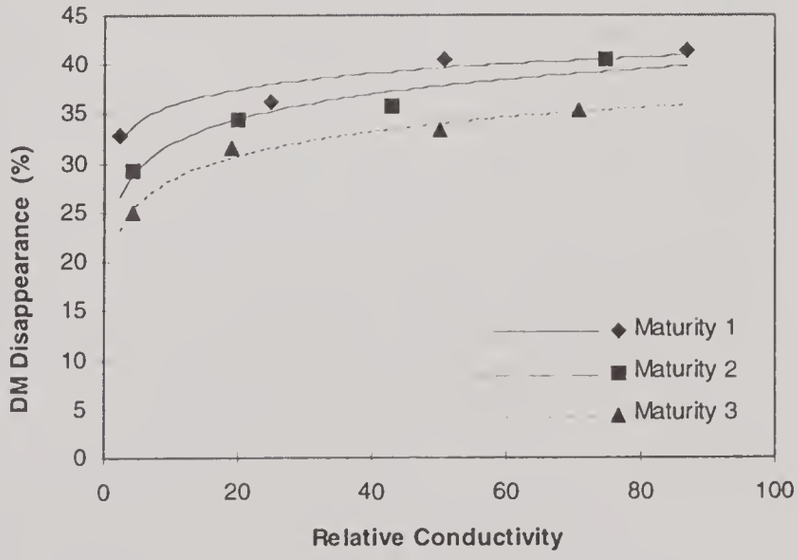


Figure 3. % DM disappearance vs. relative conductivity.

Wet Fractionation of Alfalfa Juice for Value-Added Products

R.G. Koegel and R.J. Straub

Introduction

Biotechnologists at the University of Wisconsin have created transgenic alfalfa varieties which produce industrially valuable enzymes not normally produced in alfalfa. These include alpha-amylase, widely used in converting starch to sugar, and manganese-dependent peroxidase, believed to be valuable for the bio-pulping of wood in the paper industry. Much "environmentally-friendly" processing envisaged for the future depends on an abundant and inexpensive supply of enzymes. An example of this is the hydrolysis of biomass to sugars which are fermentable to ethanol, usable as a transportation fuel.

The enzymes phytase and cellulase are currently being added to alfalfa. The former allows phosphorus in the rations of monogastrics to be used more efficiently which also results in less phosphorus loading of the environment. The latter is important for the hydrolysis of ligno-cellulosics to fermentable sugars in the production of ethanol.

Target enzymes will be harvested from the juice of transgenic alfalfa. This juice contains two forms of protein: particulate (or chloroplastic) and soluble (or cytoplasmic). Since the target enzymes generally occur with the soluble protein fraction, maximizing the yield of this fraction is of interest. In addition, the soluble fraction has potential as a protein fortifier in food products. The chloroplastic fraction contains pigmenting agents, called xanthophylls, which are valued in the poultry industry to color yolks and skin. The xanthophylls in the particulate protein concentrate give it a potential value of 2½ to 3 times that of its protein value alone based on the cost of currently used pigmenting materials. The fibrous fraction, in addition to being valuable as a ruminant feed, also has potential as a biofuel either via direct combustion (≃ 8000 BTU/lb DM) or by conversion to ethanol.

For estimating the economic potential of the wet fractionation process, it is necessary to know approximate yields of the various products.

Methods

Four 15′ x 30′ plots were laid out in an alfalfa field which had been seeded the previous year. The four plots were harvested May 22, May 29, June 8, and June 16, respectively. Each plot was subsequently cut at intervals of 30-36 days with the exception of the fourth cuttings which were delayed to allow the plants to build root reserves.

Herbage from each cutting was prepared in a rotary impact macerator and juice was expressed in a 6″ diameter Rietz Screw press.

Results

Dry matter yields for both the herbage and the juice are given in Table 1. The average seasonal dry matter per acre yields for herbage and juice were 6.02 tons and 1.89 tons, respectively, for an overall juice/herbage yield of 0.31.

The fiber fraction of plot 3, first cutting, had the following analysis: protein 9.9%; NDF, 67.7% and ADF, 60.0%, indicating high level of solubles extracted in the juice.

If the juice-derived particulate protein concentrate (~ 45% protein) and the soluble protein concentrate (~ 90% protein) yields are approximately .41 and .15, respectively (Koegel and Straub 1994) of the juice dry matter, the seasonal per acre yields of the concentrates would be .077 tons and .28 tons, respectively, and the protein yields would be 0.35 tons and 0.26 tons, respectively, for a total protein yield of 0.61 tons. If yield targets for the target enzymes of 1 - 5% of the soluble protein were attained, then 5.2 - 26.0 lb per acre of enzymes could be "harvested".

The per acre energy content of the fibrous fraction would be about 66 million BTU. If converted to electricity at an efficiency of .33, this would result in 6385 KWh or power of 100KW for over 2½ days.

Conclusions

Since the yield results shown are for a single set of plots for one year, these would be considered highly tentative. Staging the cutting of plots over a period of 4½ - 5 weeks to allow better utilization of equipment in a field-scale operation appeared promising in 1995.

Since relatively aggressive processing was used for juice expression, the juice dry matter yield of 0.3 herbage dry matter is probably close to the maximum attainable (unless rewetting and repressing is used).

Reference

Koegel, R.G. and R.J. Straub. 1994. Fractionation of alfalfa for food, feed, biomass, and enzymes. ASAE Paper #946010.

Table 1: Dry matter yields for alfalfa herbage and juice - 1995. ( ) = cutting date.

	Cutting 1		Cutting 2		Cutting 3		Cutting 4		Season		
Plot	Herbage DM (t/acre)	Juice DM / Herbage DM	Herbage DM (t/acre)	Juice DM / Herbage DM	Herbage DM (t/acre)	Juice DM / Herbage DM	Herbage DM (t/acre)	Juice DM / Herbage DM	Herbage DM (t/acre)	Juice DM (t/acre)	Juice DM / Herbage DM
1	1.42 (5/22)	.34	1.31 (6/22)	.32	1.62 (7/28)	.34	1.17 (9/1)	.34	5.52	1.83	.33
2	1.99 (5/30)	.30	1.41 (6/29)	.30	1.38 (8/3)	.33	1.03 (9/11)	.37	5.81	1.82	.31
3	2.45 (6/8)	.31	1.34 (7/10)	.32	1.29 (8/14)	.31	0.79 (10/18)	.38	5.87	1.88	.32
4	3.26 (6/16)	.25	1.80 (7/20)	.30	1.18 (8/24)	.31	0.65 (10/18)	.47	6.89	2.02	.29
								Avg.	6.02	1.89	.31

Ensilability of Whole-Plant Soybeans

R.E. Muck, R.R. Smith and T.E. Devine

Introduction

In drought years, forage supplies are often limited. One means of increasing forage supplies for dairy farms is to grow soybeans, harvesting the whole plant for ensiling. Recently, Dr. T.E. Devine with ARS in Beltsville, MD has developed soybean lines that would be grown specifically for forage production. However, little information is available about the ensilability of whole-plant soybeans.

Methods

Three entries of soybeans [PA 10-1-2 (PA), OR 5-12-1T (OR) and FS 2602 (FS)] were grown in triplicate in a randomized block design. The first two entries were experimental forage lines from Dr. Devine and the latter a commercially available variety. Plots consisted of three 6 m rows spaced 75 cm apart and seeded at 14 seeds/m. A portion of the center row of each line was harvested by hand at approximately early pod (August 1), 50% pod filled (August 23) and physiologically mature (September 27) stages for the standard soybean variety (FS). Plants were wilted in a greenhouse to approximately 35% DM, chopped with a stationary chopper, inoculated with lactic acid bacteria at 10,000 bacteria/g soybeans (to ensure adequate numbers of fermenting bacteria), and ensiled in pint canning jars.

Each entry was staged at harvest. In addition, initial samples were taken for determination of pH, dry matter, nitrogen fractions, sugar content and buffering capacity. Silos were opened after 30 d ensiling. Silages were analyzed for pH, dry matter, nitrogen fractions and fermentation products.

Results and Discussion

Total analysis of the samples is not yet complete, but sufficient results are available to indicate trends. Table 1 provides a summary of silage quality for the three entries in each of the three harvests. At the earliest harvest, only OR had reasonable silage quality. The other two lines had high pHs and low levels of lactic acid relative to acetic acid. Although no butyric acid was present, pH and dry matter contents of the PA and FS silages were such that clostridial growth was possible. Hand harvesting, which would reduce soil contamination, and the short fermentation period most likely contributed to the lack of a clostridial fermentation in the PA and FS silages. Silage quality was improved in the later harvests, with higher lactic-to-acetic acid ratios and lower pHs across entries. Fermentation characteristics were similar to those typically found in alfalfa silage although pHs were somewhat high.

Within a harvest, there were few statistical differences in silage quality among entries. In the first harvest, OR had a significantly higher lactic acid content and lower pH than the other entries. In the second harvest, FS was significantly lower in acetic acid and higher in ethanol content. In the third harvest, FS silages were higher in dry matter, ethanol and butyric acid contents and lower in soluble nonprotein nitrogen, lactic and acetic acids. The substantial differences in the third harvest between the commercial and forage entries were most likely caused by differences in maturity.

Over the three harvests, OR had the best and most consistent fermentation characteristics with the lowest pH and highest level of lactic acid. With the exception of a low soluble nonprotein nitrogen content, FS had the poorest characteristics, having the highest ethanol content and a significant butyric acid content in the third harvest.

Conclusions

The two forage entries and the commercially available soybean variety ensiled well in the later harvests (August 23 and September 27). Only the one forage entry (OR) produced a silage of good quality in the earliest harvest (August 1). Because of the higher pHs in these silages in general, it will be important that soybeans be wilted to approximately 35% DM prior to ensiling in order to prevent clostridial fermentation.

Table 1. Characteristics of 30-day, whole-plant soybean silage ensiled at three maturities.

Variety	Stage	DM	pH	Sol. NPN*	Lactic acid	Acetic acid	Butyric acid	Ethanol
		%		% DM	% DM	% DM	% DM	% DM
Aug 1								
PA	R1	32.1	5.60	8.2	2.87	3.45	0.00	0.54
OR	R1	35.9	5.17	9.1	5.16	3.12	0.00	0.82
FS	R2	37.8	5.88	9.8	2.40	3.16	0.00	0.89
Aug 23								
PA	R2.7	31.1	5.29	8.1	4.37	3.42	0.00	0.80
OR	R3.3	31.6	5.16	7.4	5.02	3.14	0.00	0.84
FS	R5.7	32.7	5.22	9.1	4.85	2.56	0.00	1.71
Sep 27								
PA	R3	30.3	4.93	9.6	6.21	2.99	0.00	0.91
OR	R4	34.3	4.86	8.0	5.97	2.56	0.00	0.82
FS	R6	38.6	4.96	4.4	3.00	1.21	0.82	1.13

*Soluble nonprotein nitrogen.

Effects of Breeding for Quality on Alfalfa Ensilability

R.E. Muck and R.W. Hintz

Introduction

Alfalfa is a forage that was once considered difficult to ensile because of its high buffering capacity and low sugar content. However, over the past 30 years alfalfa has been increasingly ensiled rather than stored as hay. The resolution to the problem was wilting the crop sufficiently so that detrimental microorganisms were inhibited by the lower moisture content of the crop as well as by fermentation acids and reduced pH. Tower silos and wilting the crop to at least 40% DM permitted good preservation and promoted the ensiling of alfalfa. Today, more farmers are adopting lower cost methods of ensiling: bunker silos, bags and wrapped bales. In these silos, alfalfa is ensiled at 30 to 40% DM, increasing the opportunity for poor fermentation. In addition, considerable efforts in the seed industry are directed at improving alfalfa quality. It is unclear if these strides to improve quality will adversely affect ensiling. If they do, the newer varieties and wetter silages of today could cause farmers considerable problems. Thus, the objective of this study was to determine if alfalfa lines bred for quality were more difficult to ensile.

Methods

Eight alfalfa genotypes were established in 8 replicate plots of each in a randomized block design. Four plots were harvested for ensiling at early bud on May 19, and the remainder were harvested at first flower (June 6). In July, regrowth was harvested from both sets of plots. Second regrowth was harvested from the first four plots at early bud on July 13. First regrowth was harvested at first flower on July 6 from the second set of plots. In all harvests, alfalfa was wilted to 35% DM in a greenhouse, chopped in a stationary chopper, inoculated with lactic acid bacteria at 10,000 bacteria/g alfalfa and ensiled, two laboratory silos per plot. At ensiling, samples from each plot were collected for analyzing pH, DM, neutral detergent fiber (NDF), acid detergent fiber (ADF), *in vitro* true digestibility (IVTD), reducing sugars, buffering capacity, nitrogen fractions and morphological stage (mean stage by weight; Kalu and Fick system). After 30 d ensiling, silos were opened and samples taken for analysis of pH, DM, NDF, ADF, IVTD, fermentation products and nitrogen fractions.

Results and Discussion

The quality characteristics [crude protein (CP), NDF, ADF and IVTD] of the initial forage varied significantly ($P < 0.05$) by genotype (Table 1). The trend of variation was as expected with the high quality lines having higher CP and IVTD and lower fiber contents than the standard lines. There was also significant variation in stage with genotype. However, the most immature and most mature lines were high quality lines, and the standard lines were of intermediate maturity on average. Other factors such as DM content, pH, soluble nonprotein nitrogen (NPN), ammonia nitrogen (NH3), and buffering capacity were unaffected by genotype.

By contrast, all characteristics of the initial forage were significantly ($P < 0.001$) affected by cutting (primary growth *vs.* regrowth), and maturity was similarly significant except for pH ($P = 0.10$). Cutting by maturity interactions ($P < 0.05$) occurred consistently across all characteristics. The only interactions with genotype that were significant ($P < 0.05$) were with maturity for stage and with cutting for DM and NH3. Consequently, differences in initial characteristics among genotypes were generally consistent across harvests.

Characteristics of the silages averaged across harvests are shown in Table 2. There was significant variation ($P < 0.05$) across genotypes for DM, pH, CP, NH3, lactic and acetic acids. However, with the exception of CP, the high quality lines contained both high and low values for each of these constituents. It should also be noted that, despite the significant differences across genotype, the ranges of DMs, pHs, and lactic and acetic acid contents were small and most likely of little practical significance.

Cutting significantly affected all silage characteristics in Table 2 with the exception of NPN. Maturity affected all but NPN and acetic and butyric acids. There were few interactions: genotype by cutting for DM and cutting by maturity for lactic and acetic acids.

Conclusions

Alfalfa genotypes varying in quality produced silages with significantly different fermentation characteristics. However, the magnitudes of these differences were small and not necessarily in an adverse direction for all high quality genotypes. These results suggest that present efforts in breeding for high quality are not having a substantial negative effect on ensiling.

Table 1. Average characteristics of the eight alfalfa genotypes prior to ensiling over four harvests.

Genotype	Quality	Stage	DM*	pH	CP	NPN	NDF	ADF	IVTD	BC
Magnum III	Std.	2.68	33.7	6.36	20.5	17.1	43.9	34.9	73.6	570
Pioneer 5373	Std.	2.64	33.9	6.37	20.7	18.3	44.1	35.3	73.9	577
RFV 2000	Impr'd	2.58	34.0	6.34	22.5	19.9	40.8	32.5	76.5	579
Alpha 2001	Impr'd	2.72	34.2	6.34	23.2	17.9	41.0	32.6	77.3	574
Banquet	High	2.64	33.6	6.39	23.4	21.2	40.4	31.9	77.0	600
DK 133	High	2.71	34.1	6.35	23.5	20.1	40.1	31.8	77.6	570
WL 252 HQ	High	2.85	33.3	6.34	24.1	17.8	40.9	32.5	78.2	591
WL 322 HQ	High	2.46	33.3	6.37	24.2	16.1	40.6	32.3	78.3	591

*DM - dry matter, %; CP - Crude Protein, % DM; NPN - soluble nonprotein N, % CP; NDF - neutral detergent fiber, % DM; ADF - acid detergent fiber; % DM; IVTD - *in vitro* true digestibility, % DM; BC - buffering capacity, meq/kg DM.

Table 2. Average characteristics of the eight alfalfa genotypes after ensiling over four harvests.

Genotype	Quality	DM*	pH	CP	NPN	NH3	Lac	Ace	But	Eth
Magnum III	Std.	32.5	5.06	24.1	56.5	8.9	5.10	2.10	0.00	0.33
Pioneer 5373	Std.	32.1	5.06	24.0	57.8	8.8	4.74	2.12	0.04	0.44
RFV 2000	Improved	33.1	5.15	24.4	57.0	8.8	4.45	2.05	0.02	0.41
Alpha 2001	Improved	33.5	5.05	24.5	55.5	8.2	4.83	1.98	0.00	0.34
Banquet	High	32.8	5.09	25.2	54.6	8.8	4.70	2.09	0.00	0.28
DK 133	High	33.1	4.97	25.5	55.3	8.4	5.24	1.98	0.00	0.35
WL 252 HQ	High	32.6	5.11	26.2	55.9	8.4	4.90	2.42	0.04	0.44
WL 322 HQ	High	32.3	5.15	24.9	57.6	9.2	4.84	2.31	0.00	0.35

*DM - dry matter, %; CP - crude protein, %; NPN - soluble nonprotein N, % CP; NH3 - ammonia N, % CP; Lac - Lactic Acid, % DM; Ace - Acetic Acid, % DM; But - Butyric Acid, % DM; Eth - Ethanol, % DM.

Effect of Storage System and Dry Matter Content on Composition of Alfalfa Silage

N.D. Luchini, G.A. Broderick, R.E. Muck, R.L. Vetter and N.F. Makoni

Introduction

It is recommended that alfalfa should be ensiled at 30 to 35, 35 to 45, or 45 to 60% DM, when using, respectively, bunker, tower, or O_2-limiting storage systems. Despite minor effects of silage DM on total CP and fiber content, DM at ensiling can cause profound changes in the N profile and fermentation products of alfalfa silage. Nutritional evaluation of forages by conventional wet chemistry procedures is expensive and time-consuming. Near infrared reflectance spectroscopy (**NIRS**) offers a rapid, low-cost method of analysis. The objectives of this study were to evaluate the effects of DM content on the composition of alfalfa ensiled using different storage systems on commercial dairy farms and to compare forage evaluation by NIRS with wet chemical analyses.

Materials and Methods

Sixty alfalfa silage samples were collected—21 from bunker, 19 from tower, and 20 from O_2-limiting silos, on 43 commercial dairy farms. Farms were located in Wisconsin and Minnesota. Sub-samples from each silo were sent to two commercial laboratories for NIRS analyses of DM, CP, ADIN, ADF, NDF, Ca, P, K and Mg. Sub-samples also were analyzed in our laboratory for DM, CP, ADF, NDF, ADIN, neutral detergent insoluble N (**NDIN**), NH_3, total AA, NPN, organic acids and ash by wet chemistry, and for pH.

Results and Discussion

Regression of DM determined by NIRS by the commercial laboratories on oven DM yielded slopes of .93 and .94 and coefficients of determination (r^2) of .98 and .99, indicating that DM analyses by NIRS and at 105°C were in good agreement. However, silage contents of CP, ADF and NDF determined by NIRS and wet chemistry differed greatly; slopes from regressions of NIRS results on chemical analysis data ranged from .57 to .90 and coefficients of determination ranged from .59 to .84. The NPN content of the silages from the tower and O_2-limiting silos was lower than that of bunker silages (Table 1). The NH_3 concentration in bunker silages was higher than that in silage from the other two systems (Table 1). In all three silo types, the drier the silage the lower the NH_3 formation during storage. Regressions of ADF and NDF on DM suggested that silages with DM lower than 45% had higher concentrations of both fiber fractions. At DM higher than 50%, ADF and NDF concentrations were not correlated with DM content. Silage from bunker silos had higher ADIN concentrations than those from the other two storage systems (Table 1). Low coefficients of determination and slopes that were not different from zero indicated that DM content was not related to ADIN in silages from any of the three systems ($P > .84$). There was a positive

relationship between NDIN and DM content when silage DM was greater than 55%. Wetter silages had greater concentrations of total organic acids. Lactic acid tended to decrease with increasing DM for the tower and O_2-limiting silages. Butyric acid was not detectable in silage from any of the tower or O_2-limiting silos; however, butyric acid was present in half of the samples from bunker silos. Higher butyric and lower lactic acid concentration in the bunker silages may reflect a shift in microbial population, from lactate to butyrate producing. As expected, silages with higher DM tended to have higher pH. The wetter bunker silages also tended to have higher pH, probably reflecting the greater NH_3 content. Bunker silages had lower relative feed value (RFV) than O_2-limiting and tower silos (Table 1). The RFV of bunker silages increased with DM; however, low coefficients of determination and slopes not different from zero for both O_2-limiting and tower silages ($P > .33$) indicated that RFV was not related to their DM content. The poorer quality found for the bunker silages may be due to poorer management used in their preservation.

Conclusions

Despite a wide range of DM contents of the silages ensiled in three storage systems, bunker silages were wetter, and O_2-limiting silages were drier, than tower silages. Wetter silages stored in bunker silos had greater amounts of NPN and ADIN than bunker silages with higher DM. Bunker silages had greater amounts of NPN and ADIN and lower RFV than silages stored in tower and O_2-limiting silos. Chemical analysis of samples from commercial dairy farms indicated that silages from tower and O_2-limiting silos were of higher nutritional quality than silages from bunker silos. Important differences were found in results from analysis of silages by conventional wet chemistry and by NIRS. Thus, caution should be used when interpreting composition data obtained from NIRS analysis of alfalfa silage samples.

Table 1. Effects of storage system on chemical composition of alfalfa silage.[1,2]

Item	Bunker	O -limiting	Tower	RMSE	$P > F$[3]
DM, %	36.8[b]	[2] 54.0[a]	49.6[a]	8.9	.001
Crude protein, % of DM	19.4	20.7	19.7	2.9	.268
NPN, % of total N	62.3[a]	55.4[b]	55.0[b]	10.0	.014
NH , % of total N	13.11[a]	6.79[b]	7.14[b]	5.54	.008
Totål AA N, % of total N	32.3	32.2	33.3	9.2	.269
ADF, % of DM	40.5[a]	34.9[b]	35.9[b]	4.1	.001
NDF, % of DM	45.8[a]	41.5[b]	41.8[b]	5.9	.020
ADIN, % of total N	9.74[a]	6.67[b]	6.78[b]	2.04	.001
NDIN, % of total N	14.1	15.0	12.2	5.9	.626
NDIN - ADIN, % of total N	4.37	8.34	5.46	4.67	.122
Total organic acids, % of DM	8.91[a]	4.75[b]	6.66[b]	3.07	.003
Succinate, % of DM	.36[a]	.19[b]	.27[ab]	.24	.050
Lactate, % of DM	3.67[ab]	2.86[b]	4.42[a]	1.94	.028
Formate, % of DM	.018	.049	.038	.050	.030
Acetate, % of DM	2.87[a]	1.16[b]	1.46[a]	1.21	.001
Propionate, % of DM	.265[a]	ND[4]	.012[ab]	.27	.010
Butanediol, % of DM	.41	.31	.28	.20	.048
Ethanol, % of DM	.28	.18	.15	.26	.629
Butyrate, % of DM	1.04[a]	ND[b]	.02[b]	1.02	.021
pH	4.84	4.87	4.69	.34	.077
RFV, %	121.5[b]	140.8[a]	137.9[a]	24.6	.019

[a,b]Means within the same row without a common superscript differ ($P < .05$).

[1]NDIN = Neutral detergent insoluble N, RMSE = residual mean square error, RFV = relative feed value, ND = Not detectable.

[2]Wet chemistry results from 21 bunker silos, 20 O_2-limiting silos and 19 tower silos.

[3]Probability of an effect of storage structure.

Facile New Synthesis of Coniferyl and Sinapyl Alcohols

F.H. Ludley and J. Ralph

Introduction

Synthesis of coniferyl and sinapyl alcohols on large and small scale has become important during recent years due to the usefulness of these lignin monomers in preparing synthetic lignins and lignin model compounds. In the past, more or less selective 1,2-reduction of ethyl ferulate and ethyl sinapate with lithium aluminum hydride, sodium bis(2-methoxyethyl)aluminum hydride or diisobutyl aluminum hydride (DIBAL-H) has been used. The problem with all these reactions is the moderate yields, the necessity of handling air-sensitive reagents under protective gases, and the varying amount of contaminating 1,4-reduction products. Here we describe a simple, quick and efficient synthesis of the two lignin monomers by sodium borohydride reduction of coniferyl and sinapyl aldehydes that have recently become commercially available. Although coniferyl alcohol is now available commercially, this procedure produces a cleaner and cheaper product.

Method

Coniferyl or sinapyl aldehyde (0.24 - 0.28 mmol) was dissolved in ethyl acetate (10 mL). Sodium borohydride (2 eq) was added and the mixture stirred at room temperature for 1 h. During that time a yellow precipitate formed. The mixture was then poured into water and washed once with saturated ammonium chloride solution. The organic layer was separated and dried over magnesium sulfate. Evaporation of the solvent in vacuo gave coniferyl or sinapyl alcohol as a slightly yellow oil in 94-98% yield.

The products were immediately characterized by NMR spectroscopy and showed no detectable 1,4-reduction products. A large-scale reduction performed on 5 g of coniferyl aldehyde required a reaction time of 6.5 hours but gave an equivalently clean product, which was crystallized from methylene chloride/petroleum ether to give pale yellow crystals.

Discussion and Conclusions

Sodium borohydride reduction of coniferyl or sinapyl aldehydes in ethyl acetate at room temperature offers an efficient method for preparing large or small amounts of clean coniferyl or sinapyl alcohols. The advantages of this method compared to the ones used to date are: the yields of the desired compounds are very high; the reaction products need no further purification and can therefore be used directly in following reactions; the starting materials (coniferyl and sinapyl aldehyde) are commercially available; there is no need to protect the phenolic hydroxy group as in some of the other preparation methods; and, most importantly, no 1,4-reduction products are formed. With the simplicity of this procedure and the relative inertness of the reducing agent (sodium borohydride), even a non-chemist can run the reaction and prepare quality lignin monomers.

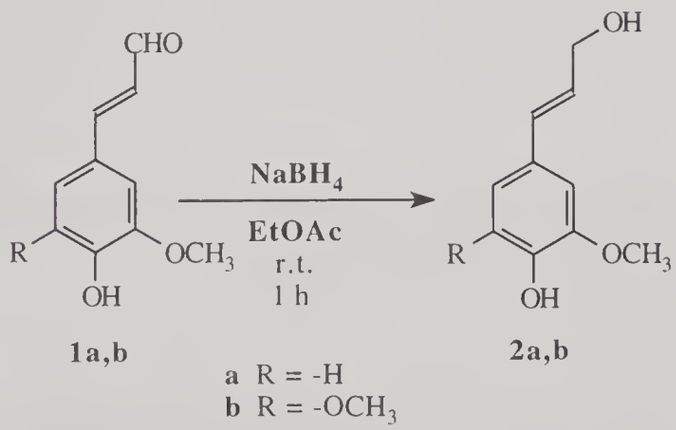


Figure 1. $NaBH_4$ reduction of coniferyl and sinapyl aldehydes.

Synthesis of *cis*-Monolignols

Y. Zhang and J. Ralph

Introduction

cis-Cinnamic acids have been found in specific plants with important physiological roles in development. *cis*-Coniferyl alcohol also has been isolated from some plants. We are interested in the role of *cis*-monolignols in plant development and the specific characteristics of lignin structure if both *cis*- and *trans*-monolignols are incorporated. The study of synthetic lignins (DHPs) from *cis*-monolignols will aid in understanding the role of *cis*-monolignols in plants. Until now, the major problem confronting the study is that *cis*-monolignols are reported to be unstable and easily isomerised to *trans*-isomers, so the pure *cis*-isomers are hard to synthesize. Here we report a new method to synthesize the *cis*-monolignols in high yield and extremely high purity.

Methods

The reaction pathway for *cis*-coniferyl alcohol synthesis is shown in Figure1. *p*-Coumaryl alcohol and sinapyl alcohol can be synthesized similarly.

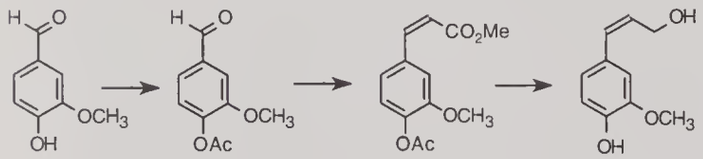


Figure 1. Synthesis of cis-coniferyl alcohol.

Results and Discussion

Traditionally the *cis*-monolignols are synthesized via photo induced isomerization of *trans*-isomers. The major problem of this method is that the resultant product is a mixture of *cis*- and *trans*-isomers from which separation is difficult. So until now, research of the role of *cis*-monolignols in lignin structure and plant development has utilized the mixture, complicating the results.

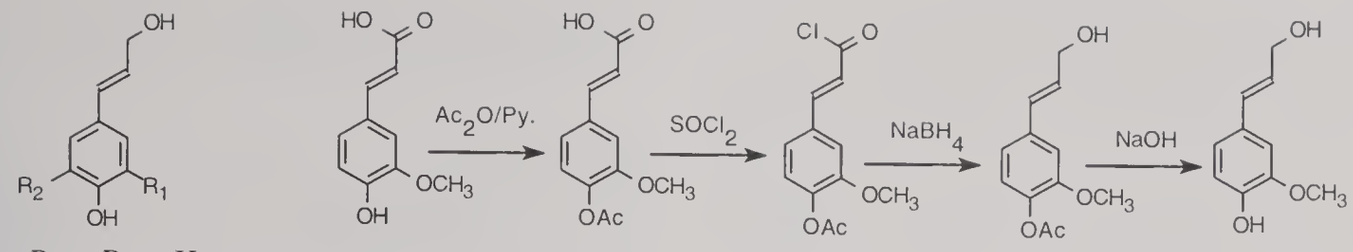
trans-Ferulate is easily constructed with vanillin and triethylphosphonoacetate via an HWE reaction. The transition state strongly favors the *trans*-configuration. Some reports have shown that the replacement of the ethyl group with trifluoro group will make the *cis*-configuration much more favorable. We figured that the transition state would still favor the *cis*-isomer using vanillin as the substrate. The reaction was indeed successful, resulting in pure *cis*-ferulate in 95% yield. The normal phenol protecting group in this reaction is an ether, but its cleavage under mild acidic conditions resulted in some isomerization. In order to avoid this drawback, acetate was used as a protecting group. The acetyl group need not be cleaved after the formation of ferulate and can be directly reduced in the DIBAL reduction step to afford the coniferyl alcohol. Acetate is not normally used as a protecting group in alkaline conditions, but in this reaction it worked very well. Additionally, the ferulate acetate is much easier to crystallize than the ether-protected ferulate. The final DIBAL reduction afforded *cis*-coniferyl alcohol; it was crystallized from acetone/water or acetone/petroleum ether in 85% yield. The *cis*-sinapyl and *p*-coumaryl alcohols are synthesized analogously with yields of 82% and 81%.

A Convenient Synthesis of Coniferyl Alcohol

F. Lu, F.H. Ludley and J. Ralph

Introduction

Lignin is a complicated natural aromatic polymer derived from enzyme-mediated dehydrogenation of 4-hydroxycinnamyl alcohols **a-c** (Fig. 1). So far a lot of effort has been expended to elucidate the lignin structure and its chemical relationships to cell wall components such as carbohydrates and phenolics. Because of their relative simplicity and their uniqueness as a tool to approach lignin structure, synthetic lignins or dehydrogenation polymers (DHPs), made in vitro by oxidative polymerization of 4-hydroxycinnamyl alcohols,



a $R_1 = R_2 = H$
b $R_1 = OCH_3$, $R_2 = H$
c $R_1 = R_2 = OCH_3$

Figure 1. Preparation of hydroxycinnamyl alcohols from acid chlorides.

have been used extensively to model lignin biosynthesis and obtain information about lignin structure. However, such investigations are usually difficult to carry out due to the poor accessibility of the 4-hydroxycinnamyl alcohols.

Several synthetic methods of 4-hydroxycinnamyl alcohols have been developed. However, all of them suffer from either low yield, undesirable contaminants, or difficult to handle reagents. Here we report a convenient synthesis of coniferyl alcohol **b** (Fig. 1), one of the most widely used 4-hydroxycinnamyl alcohols for DHPs.

Methods

A) 4-Acetoxylferulic acid **2** is prepared from ferulic acid **1** by acetylation using Ac_2O/Pyridine and crystallized from ethanol after standard work-up, 96% yield.

B) 4-Acetylferuloyl chloride **3** is made as follows: compound **2** (5 g) is suspended in toluene (120 ml) with thionyl chloride (5 ml) at 80°C for 20 min, followed by addition of a catalytic amount of pyridine. In about 10 min, a clear solution is obtained accompanied by a settling out of an insoluble oil. The supernatant is transferred to a 500 ml flask and co-evaporated with toluene several times. A pure light yellow solid chloride is obtained in 96% yield and is used directly. It can be crystallized from hot toluene and stored for extended periods.

C) 4-Acetoxylconiferyl alcohol **4**: Compound **3** (1 mmole) is dissolved in ethyl acetate (10 ml distilled), followed by addition of sodium borohydride (4.5 eq). The mixture is stirred and monitored by TLC until the reaction is complete (1.5 h). After standard work-up, 4-acetoxyconiferyl alcohol is obtained in 95% yield.

D) Coniferyl alcohol **5:** To a solution of compound **4** (1 mmole) in ethanol (15 ml) is added NaOH (2 N, 2 ml) and this solution is stirred for 2 h at r.t. under N_2. The solution is acidified with 3% HCl and extracted with ethyl acetate. Normal work-up gives coniferyl alcohol (90%) as an oil which is crystallized from ethyl acetate/petroleum ether (pale yellow crystals, yield not calculated).

Discussion

It is well known that the selectivity of sodium borohydride reduction is highly solvent dependent, although most reductions using sodium borohydride are performed in alcoholic solvent systems. Acid chlorides have been reduced to alcohols by sodium borohydride in inert solvents such as dioxane. However, in the initial studies, α,β-unsaturated acid chlorides were reduced to saturated alcohols in low yield. Sodium borohydride adsorbed on alumina was shown to reduce acid chlorides to alcohols and α,β-unsaturated acid chlorides to allylic alcohols. Sodium borohydride has been used in THF for reductions of conjugated enones and enals with variable regioselectivities. Monoacetoxyborohydride, generated in situ by addition of one equivalent of glacial acetic acid to a suspension of sodium borohydride in dry THF, has been reported to be capable of reducing conjugated enones and enals to allylic alcohols in good yields.

We recently found that sodium borohydride in ethyl acetate at room temperature is capable of converting α,β-unsaturated acid chlorides efficiently to primary allylic alcohols in high

yields. Since 4-acetylferuloyl chloride can be prepared easily from 4-acetoxylferulic acid, and that sodium borohydride is a mild, cheap and easy to handle reducing agent, it would be an attractive and convenient way to synthesize coniferyl alcohol from ferulic acid by using sodium borohydride reduction of the acid chloride **3**. In the scheme, every step involved has a high yield and is easily carried out without special precautions. This approach can also be applied to synthesis of coumaryl and sinapyl alcohols with good yield.

Conclusion

We have developed a convenient and high yield method for synthesis of coniferyl alcohol from ferulic acid. A distinct advantage of the method is characterized by using sodium borohydride which is cheap and easily handled.

Synthesis of *p*-Hydroxycinnamyl-*p*-Coumarate and Ferulate Mono- and Diglucopyranosides

F.H. Ludley and J. Ralph

Introduction

Grass lignins have substantial amounts of *p*-coumaric acid esterified, as we have recently shown (see last year's Summaries), specifically at the γ-position of lignin sidechains. This implicates the incorporation of the *p*-coumarate esters of the lignin monomers coniferyl and sinapyl alcohols in the lignin biosynthesis. However, our attempts to prepare synthetic lignins mirroring the structure of the native grass lignins have been hampered by the extreme aqueous insolubility of these esterified compounds. We reasoned that glucoside derivatives should be much more soluble, can release the esterified monomers when glucisodase enzymes are used, and may even mimic the manner in which the plant transports these compounds to the cell wall for their subsequent polymerization. Of course, synthesis of the required glucosides was far from a trivial task. One puzzling feature of grass lignins that we hope to answer using these compounds is why the *p*-coumarate unit does not become involved in the free-radical reactions of lignification; under in vitro conditions with model compounds, the *p*-coumarate enters freely into these coupling reactions.

Method

Reaction of the *p*-hydroxybenzaldehydes **1a**,**b** with 2,3,4,6-tetra-O-acetylglucopyranosylbromide **2** and potassium carbonate in acetone at room temperature gives the *p*-hydroxybenzaldehyde-2,3,4,6-tetra-O-acetyl-glucopyranosides **3a**,**b**. The compounds precipitate cleanly from a toluene/petroleum ether solution and no further recrystallization is necessary. Knoevenagel-condensation of **3a**,**b** with malonic acid in pyridine/piperidine at 100°C leads to the *p*-hydroxycinnamic acid-2,3,4,6-tetra-O-acetyl-glucopyranosides **4a**,**b**. Reacting *p*-hydroxycinnamaldehydes **5a**,**b** with 2,3,4,6-tetra-O-acetyl-glucopyranosylbromide **2** under the conditions mentioned above yields the glucosides **6a**,**b** which are reduced in very high yields to the *p*-hydroxycinnamylalcohol-2,3,4,6-tetra-O-acetyl-gluco pyranosides **7a**,**b**. All acetyl-protecting groups remain intact during this reaction; no further work-up of the products is needed. Condensation of **4a**,**b** with **7a**,**b** proceeds at room temperature in methylene chloride using dicyclohexylcarbodiimide (DCC) and 4-N,N-dimethylaminopyridine (DMAP) as catalysts and gives the final products (esters) **8a-c** in yields between 53.4 and 80.6%. The products are isolated again by precipitation out of toluene/petroleum ether, purified by column chromatography and recrystallized from 95% ethanol. All compounds were characterized directly by NMR-spectroscopy. To enhance the solubility in water necessary for the following preparations of DHP's, the acetate protecting groups are cleaved using either a solution of pyrrolidine in 95% ethanol or sodium bicarbonate in methanol. The latter method is sometimes disadvantageous because some of the compounds undergo an ester cleavage during the deacetylation reaction. Nevertheless, it is a surprise

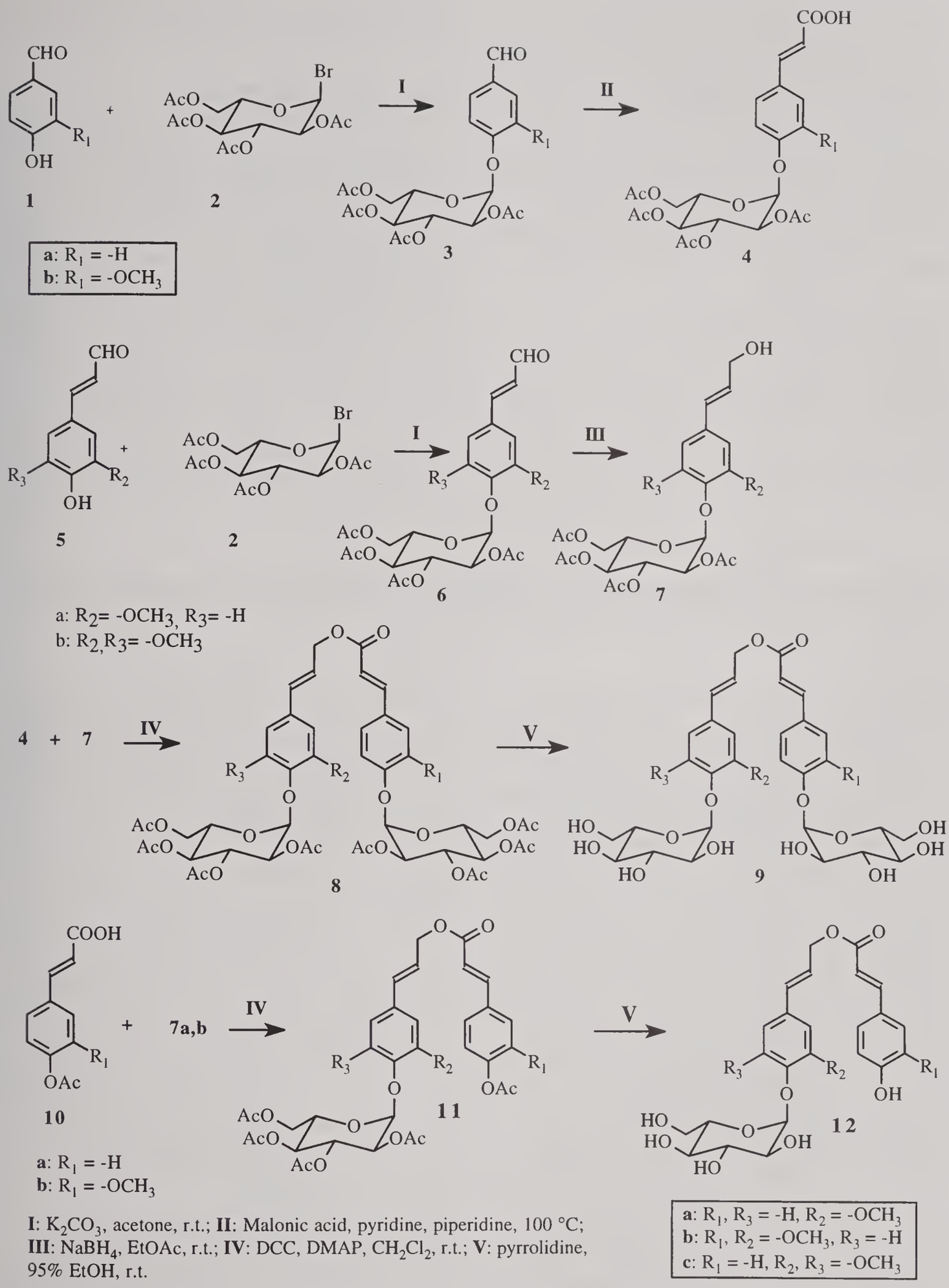


Figure 1. Synthetic scheme used to prepare hydroxycinnamyl p-hydroxycinnamate derivatives.

that such a mild reagent as sodium bicarbonate is capable of not only cleaving phenolic acetates but also aliphatic ones. It is even more interesting that it cleaves the ester bond in some of the compounds **8a-c** proving, on the other hand, that these bonds are relatively weak compared to other ester bonds. To remove any residues of acetylated material, the compounds were washed once with acetone and dried. Yields of the final compounds **9a-c** prepared during these reactions are between 83 and 98%.

The main purpose of preparing the *p*-hydroxycinnamyl *p*-coumarates and ferulates as diglucosides **9a-c** was to increase their water solubility compared to the free phenolic structures. To investigate whether the water solubility would already be high enough with only one glucoside attached, the *p*-hydroxycinnamyl *p*-coumarate and ferulate monoglucosides **12a-c** were prepared using an analogous reaction sequence.

Discussion and Conclusion

A synthetic method has been developed to prepare *p*-hydroxycinnamyl *p*-coumarate and ferulate mono and diglucopyranosides. An advantage of the reaction sequence shown is that most of the precursors and intermediate compounds can be easily isolated by precipitation from toluene/petroleum ether solutions and need no further purification. The preparation of the esters **9a-c**, as well as the deacetylation reactions leading to the final products **12a-c**, gives good yields. We shall now utilize these compounds to make synthetic lignins to mimic grass lignins and to explore why the *p*-coumarate group does not become involved in the radical coupling lignification reactions.

Preparation and Value of Synthetic Lignins with NMR-Invisible Methoxyl Groups

J. Ralph, Y. Zhang, and R.M. Ede (University of Waikato, NZ)

Introduction

NMR spectroscopy is an invaluable aid to determining details of the structures of cell wall components including lignin. And synthetic lignins, so-called DHPs, are enormously valuable to elucidate reaction pathways and provide the NMR data required to interpret spectra from real plant materials. One problem with lignin and DHP spectra is systematic artifacts that arise from various machine imperfections, vibrations of many types, and our impatience for the spectra which dictates that we run acquisitions before the poor nuclei have 'relaxed' properly. The most troubling problem is an artifact known as T_1-noise, a particular problem around the intense methoxyl peaks (in both proton and carbon dimensions of 2D spectra), where artifacts obliterate or obfuscate the data in that region. In this report, we illustrate a method to synthesize the coniferyl alcohol with an isotopically modified methoxyl group and transfer it into a DHP. This provides a rather slick method for rendering methoxyls in synthetic lignins NMR-invisible, greatly improving the appearance and interpretability of NMR spectra.

Methods

Coniferyl alcohol with NMR-invisible methoxyl group was synthesized as shown in Fig. 1. It was biomimetically converted into DHPs by established methods. 2D NMR methods were essentially standard Bruker versions.

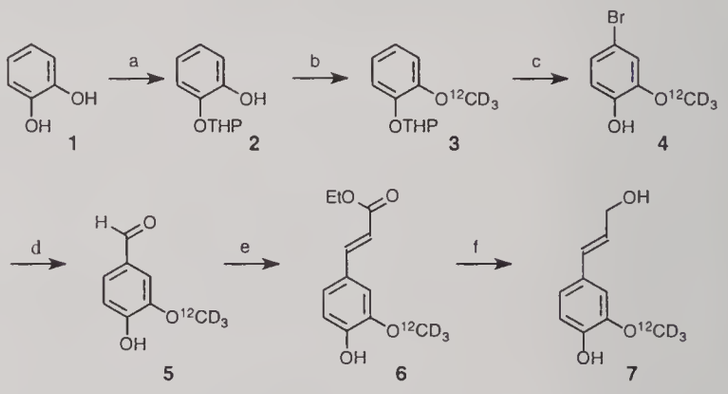


Figure 1. Synthetic scheme to produce coniferyl alcohol with an NMR-invisible methoxyl group.

Results and Discussion

An elegant solution to the methoxyl region T_1-noise artifact problem is possible for synthetic lignins; for real lignins, this is not possible but, as noted in an accompanying report, there is also an

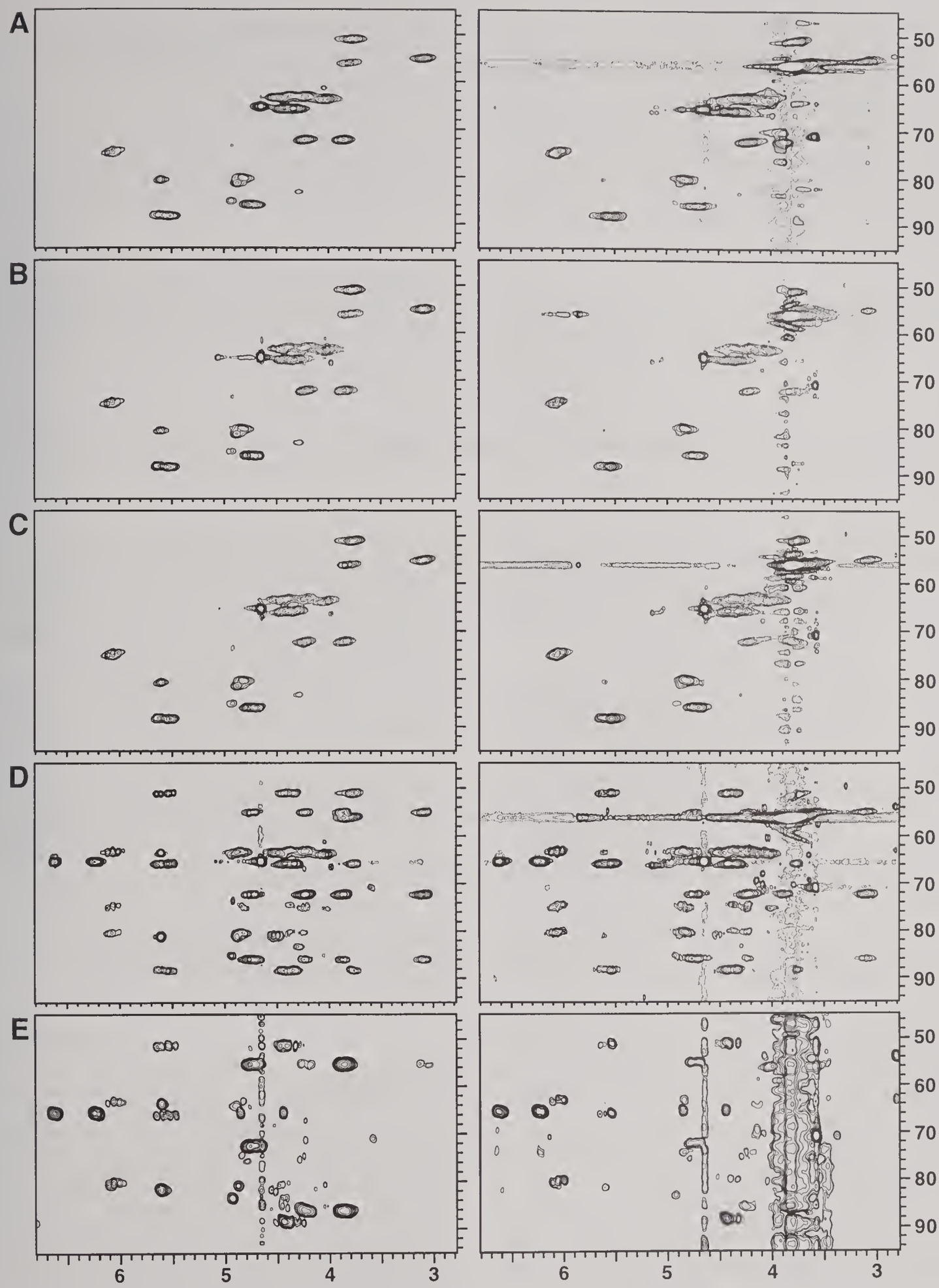


Figure 2. 2D NMR spectra of synthetic DHPs made using (left) normal coniferyl alcohol and (right) 'methoxyl-less' coniferyl alcohol. A) HMQC, B) DEPT-HMQC with a π/3 editing pulse (CH's up, CH_3's and CH_2's down), C) DEPT-HMQC with a π editing pulse (CH's and CH_3's up, CH_2's down), D) HMQC-TOCSY, E) HMBC.

instrumental solution. The idea for synthetic lignins is to make the methoxyl group NMR-invisible, but normal in every other regard. That can be done by replacing the normal methoxyl carbon (which has *ca* 1% natural abundance of the NMR-active ^{13}C) by the ^{12}C isotope (^{13}C-depleted) which is not NMR-active. Replacing the protons with deuterons also removes the methoxyl from the proton spectrum.

As shown in Fig. 2, a variety of 2D NMR spectra of a DHP produced from the specialized coniferyl alcohol **7** are spectacularly superior to an equivalent DHP made from normal coniferyl alcohol. The spectra also illustrate the value of spectral editing in 2D NMR experiments that has not yet attracted much attention from lignin chemists. The DEPT-HMQC experiment can aid in assignment by selectively inverting (for example) -CH_2 resonances (Fig. 2C).

The freedom from artifacts in these spectra will allow more detailed assignments to be made and aid in the characterization of real plant lignins.

Incorporation of 5,5-Coupled Diferulate Into Lignins

S. Quideau and J. Ralph

Introduction

In past Research Summaries, we have presented studies that produced compelling evidence that plant cell wall ferulates actively incorporate into lignins via radical processes, rather than by an 'opportunistic' mechanism that has been widely held. Finally, last year, two-dimensional NMR spectra from a uniformly ^{13}C-enriched ryegrass provided unambiguous proof that this was indeed occurring in plants. Even more importantly, and somewhat unexpectedly, the NMR spectra also revealed that ferulates were acting as nucleation sites for the lignification process and therefore play a crucial role in directing the growth and development of the plant cell wall, ultimately impacting its digestibility. Ferulate dimers were also shown to be far more important in the cell wall than had been thought — prior work has neglected most of the diferulate isomers and identified only the 5,5-coupled dimer. Ferulate dimers, diferulates, are particularly important in the wall since they are capable of simultaneously cross-linking polysaccharide chains to each other and then to lignin, effecting powerful wall cross-linking.

Unfortunately, with the sole 5,5-diferulate that had been reported and quantitated for the past 20 years, it has been assumed to cross-link to lignin via the same old 'opportunistic' mechanism. Although it is almost absurd now to think that any diferulate will not also become actively involved in the free radical coupling processes that occur during lignification, there were still questions about what products would result from such radical coupling. Unfortunately, directly observing the fate of diferulates from NMR of plant isolates is an even more foreboding task than identifying the role of ferulates because of the number of ferulate dimers, their lower levels, and the problem that the ferulate cross-linked to lignin in a dimer is difficult to resolve from the structurally similar ferulate-lignin cross-link itself. Consequently, in a manner similar to that used previously to identify how ferulate could be incorporated into lignin, strategically ^{13}C-labeled 5,5-diferulate was biomimetically incorporated into a synthetic lignin and its incorporation profile determined by NMR methods.

Experimental

A ^{13}C-labeled model compound (Fig. 1) which mimics the 5,5-diferulate as it is attached to the C5-position of arabinofuranosyl residues on arabinoxylans in grasses was synthesized by a route far too complex to be described here. At the 5% level, it was biomimetically incorporated into a synthetic lignin from coniferyl alcohol. 2D-long range correlation (HMBC) spectra of the lignin-diferulate complex in 9:1:1 acetone-d_6:D_2O:dmso-d_6 were run to determine the incorporation profile

(the structures produced by incorporation of the diferulate into the lignin).

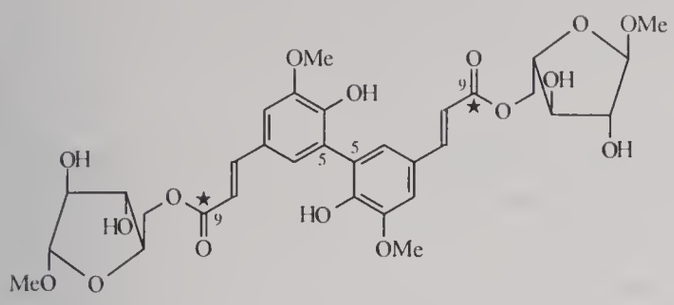


Figure 1. The ^{13}C-labeled 5,5-diferulate dimer which mimics the structure in arabinoxylans.

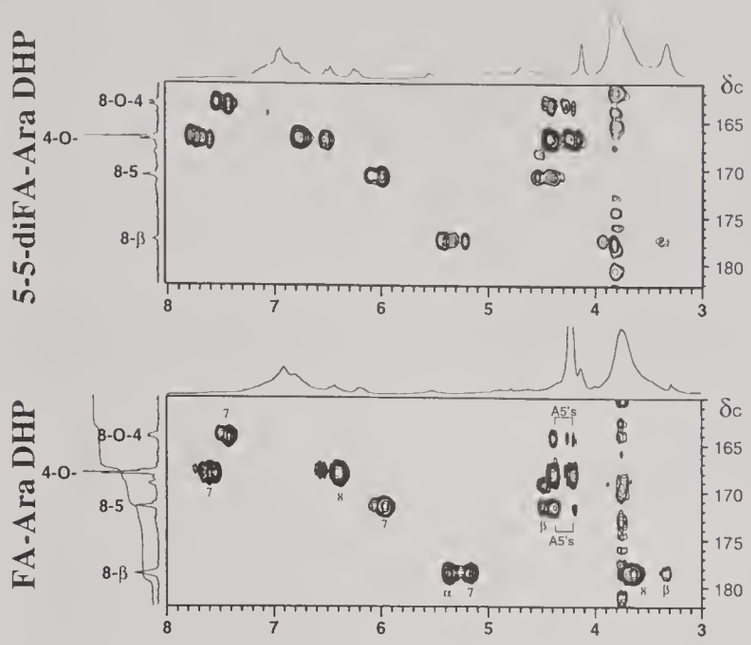


Figure 2. HMBC subspectra (carbonyl region only) of the product resulting when the labeled diferulate model (upper) or the corresponding ferulate model (lower) is biomimetically incorporated into a DHP. Similar incorporation profiles are seen. Radical coupling to 8-O-4', 4-O-X' (including phenylcoumarans), 8-5' and 8-β' products are readily identified.

Results and Discussion

In addition to 4-O-coupled products that can arise from either mechanism, diferulate produced 8-coupled products (8-O-4', 8-5', and 8-β') that can only arise by the radical mechanism. The incorporation profile, Fig. 2, is strikingly similar to that of ferulate. Clearly, diferulates and ferulates can each undergo radical coupling into lignin to produce the full range of expected structures. It is important to note that only the 4-O-products are capable of releasing identifiable ferulate or diferulate and this becomes particularly complex for diferulate which has two (joined) ferulates which may incorporate somewhat independently. Clearly, as we have warned and demonstrated with ferulate, 5,5-coupled diferulate (and in fact all of the isomeric diferulates) are, and will continue to be, severely under-quantitated because it is impossible to release them from their intimately associated lignin. They therefore have a greater role in cell wall cross-linking and consequent indigestibility than is usually assumed.

An Unusual Lignin From Kenaf

J. Ralph, R.D. Hatfield, H.G. Jung (and J.S. Han and S.A. Ralph, U.S. Forest Products Laboratory).

Introduction

In a collaborative project with the U.S. Forest Products Laboratory, we have been examining the structure of the lignin from kenaf, a dicot. Although it does not have much to do with forages, the novel lignin is helping us to assign and determine more about the structure of lignins in forage grasses and legumes. Lignins have been characterized from such a variety of plant materials that it seems unlikely for a new type of lignin to be revealed. The lignin from kenaf is, however, strikingly different from any other lignin that has been characterized. Its uniqueness is revealed both in terms of its high syringyl content and in the extensive acetylation of the side-chain hydroxyls.

Experimental

Kenaf lignin was isolated by traditional procedures from bast fibers from the core of Tainung Kenaf stems. NMR spectra were run on the Center's Bruker AMX-360.

Results and Discussion

The kenaf lignin was initially intriguing because of

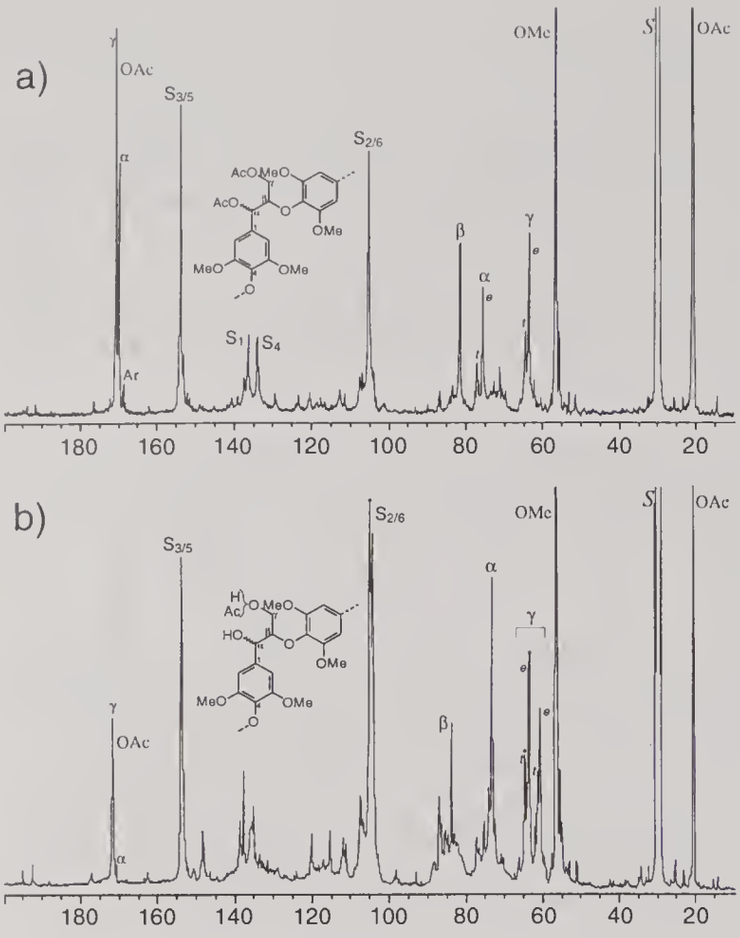


*Figure 1. ^{13}C NMR spectra of the isolated Kenaf lignins showing the preponderance of syringyl peaks. Assignments are for β-ethers: a) acetylated, b) underivatized, * indicates easily identifiable peaks that result from acetate substitution.*

its high syringyl content. Dicots typically have a rather even syringyl:guaiacyl distribution, whereas nitrobenzene oxidation of the kenaf dioxane lignin gave a molar syringaldehyde:vanillin ratio of 6.0 (without loss corrections). Syringaldehyde:vanillin ratios in hardwoods have been reported up to *ca* 5.2 and a thesis describes an 8.4 ratio. The dominance of syringyl units in this kenaf leads to a very simple lignin as seen from the ^{13}C-NMR of the acetylated kenaf dioxane lignin, Fig. 1a. What is striking, in addition to the obviously high syringyl:guaiacyl ratio, is that β-ethers predominate significantly over other interunit linkage types (cf. the β-β units at *ca* 55.5 ppm), and that the material is highly etherified (since the proportion of phenolic endgroups, seen from the aromatic acetate carbonyl peaks, is low). These values have been estimated from quantitative ^{13}C NMR at ~80% β-ether units and 14% phenols. The aliphatic region of the 2D HMQC-TOCSY spectrum, Fig. 2, confirms the assignment of sidechain protons, again shows that syringyl β-ether units predominate, and beautifully disperses the isomers. The prior observations that *erythro* β-ether isomers predominate in syringyl units is strikingly revealed in this spectrum; there is little diastereoselectivity in guaiacyl lignins.

The NMR spectrum of the un-derivatized lignin, Fig. 1b, provided even more startling information — the lignin was extensively acetylated (*ca* 50%). The spectrum shows that acetylation was almost entirely (*ca* 95%) at the primary γ-position of the sidechain, as was confirmed by an HMBC experiment correlating the acetate carbonyl carbon with γ-protons on lignin (not shown). Although acylation of dicot lignins by hydroxycinnamic acids has previously been reported to be almost insignificant, grasses have proportions of sidechain alcohol groups esterified by *p*-coumaric acid; such esterification has recently been shown to be exclusively at the γ-position in a maize lignin isolate and indications are that other grasses, both C_3 and C_4, imply that grasses pre-esterify lignin monomers to produce the hydroxycinnamyl *p*-coumarates which are then exported and incorporated into a lignin complex by the traditional oxidative coupling mechanisms. Presumably, the same can be concluded in the case of the kenaf in this study; the acetate is not so cleanly at the γ-position (*ca* 95%), but we have previously observed acetate migration in lignin model compounds. We suspect that, in kenaf,

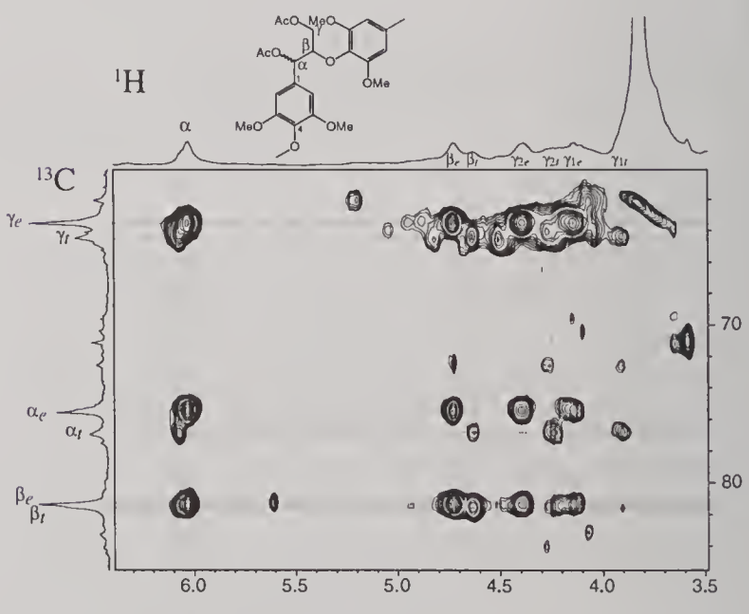


Figure 2. Aliphatic region of a 2D HMQC-TOCSY experiment showing syringyl β-ether units and the excellent dispersion of erythro- and threo-isomers.

sinapyl (and perhaps coniferyl) acetates are enzymatically produced and exported for lignification with sinapyl and coniferyl alcohols. It is most likely that the small amount of α-acetate derives from internal migration rather than from the alternate mechanism of acetate addition to quinone methide lignin intermediates, a mechanism over which the plant has no direct control. The presence of such large amounts of acetate on lignin was sufficiently novel that our procedure for the lignin isolation came under close scrutiny. The isolation was repeated under conditions in which no reagents or solvents could conceivably produce acetylation artifacts; the lignin isolated in this manner retained the extensive acetylation.

We are currently intrigued by what might be the function of such an acetylated lignin for the plant, and if somehow (and for some reason) this is a mechanism to achieve high syringyl lignins — syringyl rich synthetic lignins with high molecular weights are difficult to prepare in vitro. Of practical relevance is that the presence of acetate groups on kenaf lignin will consume base during alkaline delignification, but that delignification of this lignin fraction should be relatively straightforward and extensive because the high-syringyl lignin is relatively unbranched and, as seen from the ^{13}C-NMR spectra, contains a substantial proportion of high-temperature-base-cleavable β-aryl ether units.

In summary, kenaf lignin is unusual and perhaps unique. It actually represents a new lignin type implicating new monomers (the 4-hydroxycinnamyl acetates) in its synthesis. The NMR spectra, being relatively simple and syringyl rich, are providing data for syringyl residues that we did not have and are therefore valuable in aiding assignment and structural elcuidation of more traditional forage lignins.

A New Method for Lignin Characterization (Preliminary Report)

F. Lu and J. Ralph

Introduction

Unlike other natural polymers, such as proteins, polysaccharides and nucleic acid which have interunit linkages susceptible to enzymic and chemical hydrolyses, lignin contains non-cleavable carbon-carbon and diphenyl ether bonds between its building units. So only a part of lignin, where units are linked by β-O-4 or α-O-4 ethers, can be degraded and characterized. If uncondensed linkages are cleaved selectively and completely, the characterization of the degradation products such as monomers, dimers and trimers will provide valuable structural information about the initial lignin. At present, thioacidolysis is the preeminent method for quite selectively cleaving ethers to produce analyzable monomers and dimers. That procedure, while valuable, is not a trivial technique to perform and has significant drawbacks, not the least of which is the foul stench of the required reagents.

Here we report on a completely new approach to complete ether cleavage in lignin to provide a relatively simple, efficient method for lignin analysis. Our results with some representative lignin models, isolated lignin samples, and plant materials demonstrate the high selectivity, high yield of the degradation products and simplicity in operation as well as the usefulness for lignin structure characterization. Regrettably, the procedure is not completely developed at this point but, as it is intended for release at the March National American Chemical Society Meeting in New Orleans, belongs here this year.

Experimental

[Not the final procedure!] To 10 mg of lignin model or lignin sample in 10 ml flask is added 2 ml of stock reagent (AcBr in acetic acid). The mixture is kept at room temperature with gentle stirring overnight. After removal of solvent by rotary evaporator(< 45 °C), the residue is dissolved in 2

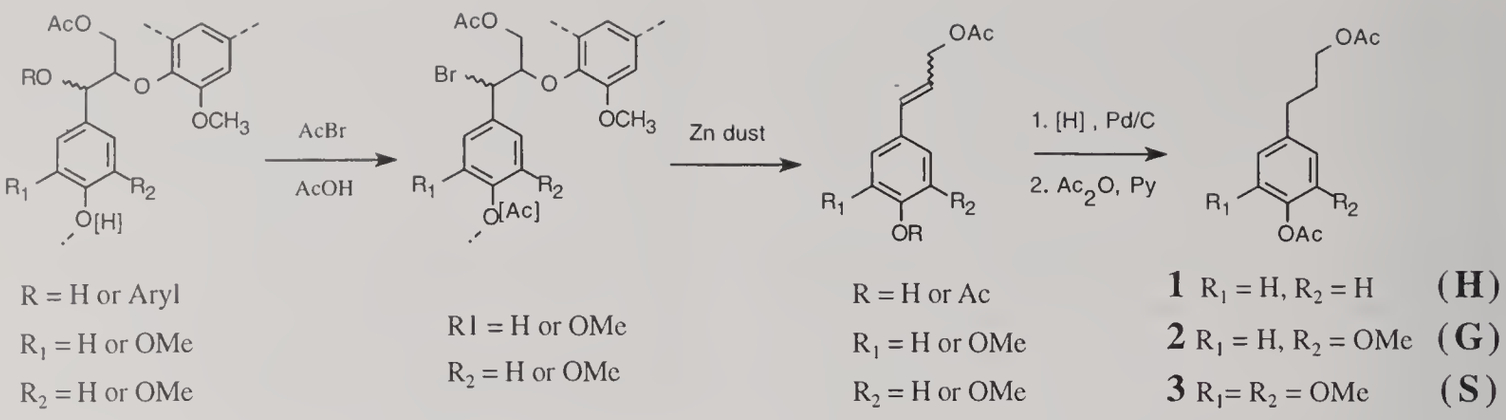


Figure 1. AcBr/Zn-based method for cleavage of ether linkages in lignins to produce monomers 1-3.

ml of dioxane to which is added 50 mg of Zn dust while it is well stirred, followed by addition of 2 ml of 0.8 N HCl 4:1 dioxane water solution. This mixture is stirred for 15 min. After addition of internal standard (methyl 3,5-dimethoxybenzonate) the mixture is poured into ethyl acetate (20 ml) and washed with 3% of HCl twice, sat. NH_4Cl once and dried over $MgSO_4$. After evaporation the residue is dissolved in 3.0 ml of acetic acid to which 10 mg of $Pd/BaSO_4$ is added. This mixture is stirred for 2 h under hydrogen (balloon), then acetic acid is removed by evaporation and 1 ml of pyridine/Ac_2O (1:1) is added. After standing for 45 min. and normal work-up, the residue is dissolved in 200 µl of methylene chloride and 1 µl of this solution is injected into GC for analysis.

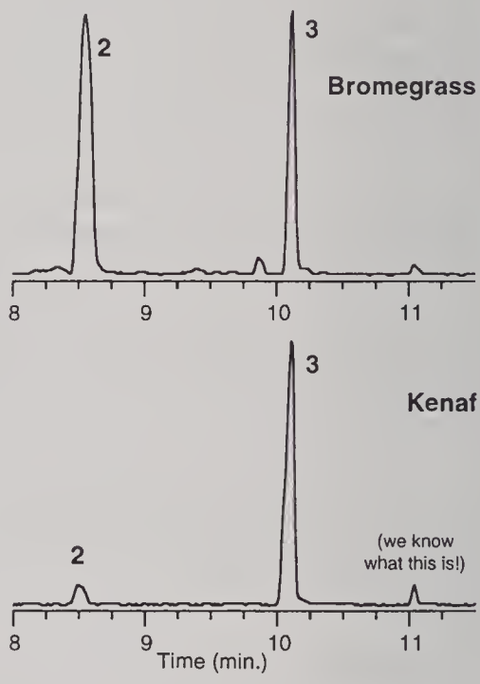


Figure 2. GC-MS total ion chromatograms of the products from two isolated lignins.

Table 1. Yields of the main monomers by the method from lignin models and MWLs.

Model or Lignin Sample	Yield (% W)[a]	Molar Yield[a] (mmole/g)	Relative ratio H : G : S
Guaiacyl β-O-4	95[b]	——	——
Syringyl β-O-4	91[b]	——	——
Pine	19.14	722	1 : 13.5 : 0
Willow	21.84	904	1 : 11.7 : 12.6
Kenaf	30.50	1050[c]	trace : 1 : 5
Bromegrass	20.35	781	1 : 19.6 : 15.6
Alfalfa	13.62	570	1 : 10.4 : 4.1
Corn	12.96	351	1 : 6 : 5.7

[a]The yield is based on whole MWLs.
[b]Theoretical yield.
[c]The molar yield from kenaf lignin by thioacidolysis is 967mmole/g.

Results and Discussion

As shown in Figure 1, AcBr/Zn degradation method includes three key steps: 1. bromination and acetylation with AcBr, 2. reductive elimination with Zn dust, and 3. hydrogenation and acetylation. The reaction of lignin with AcBr in acetic acid results in complete dissolution of lignin (and other wall components) and formation of bromo-

acetylated lignin derivative which has β-bromoaryl ether skeleton. In the next step, the β-bromo ethers are cleaved by reductive elimination with Zn forming pairs of cinnamyl acetate isomers. It is necessary to have the last step to reduce the numbers of degradation monomers and make GC quantitation simpler. Thus the monomers derived from this method are essentially **1-3** (see Figure 2). From the table below we can see that β-ether linkage in model compounds, representatives of lignin major substructures, is efficiently cleaved by this method and monomers are recovered in high yield (95% and 91% of theoretical). When isolate lignins are used in this method, substantial amounts of degraded monomers are obtained; the yield of the monomers from kenaf lignin is higher than from thioacidolysis.

In summary, we have developed a new method which selectively cleaves ether linkages in lignin, a useful lignin characterization method. Its relative simplicity, selectivity (cleanliness) and the use of relatively innocuous reagents, may well challenge the current thioacidolysis procedure.

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^{13}C NMR Studies on a New Method for Lignin Characterization

F. Lu and J. Ralph

Introduction

Despite great progress in lignin chemistry, precise elucidation of lignin structure is still a challenge for chemists. Recently we developed a simple and powerful method which selectively and efficiently cleaves α-ether and β-ether linkages to allow quantitative analysis of lignin structural units involved in uncondensed structures (see preceding paper). In this paper we report our investigation using NMR techniques to show the high selectivity, high yield conversions involved in the method.

Experimental

The analysis procedure is described in the preceding summary. ^{13}C NMR spectra of lignin models and lignin derivatives in acetone-d_6 were recorded under standard small-molecule conditions on a Bruker AMX-360 instrument; the central solvent peaks were used as internal reference (^{1}H, 2.04 ppm; ^{13}C, 29.80 ppm).

Discussion

Our method includes three key steps: bromination, two-electron reductive cleavage, and hydrogenation. The ^{13}C NMR spectra of acetyl bromide treated model compounds (**A** and **C**) and milled wood lignins (**B** and **D**) are shown in Fig. 1. β-Aryl ether models are quantitatively converted to acetylated α-bromides as shown in Fig. 1 (**A**, **C**) and β-aryl ether substructures in lignins are also

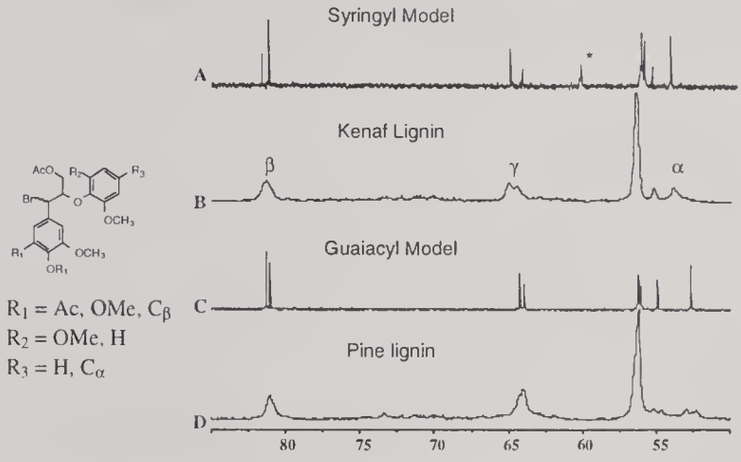


Figure 1. Sidechain region of ^{13}C NMR spectra of AcBr treated lignin models and lignins: A, syringyl model; B, kenaf lignin (an S-rich S/G lignin); C, guaiacyl model; D, pine lignin (a G-lignin).

converted to the corresponding derivatives which have the same characteristic peaks (C_α, C_β and C_γ are shown in Fig. 1). It is also seen that the major linkage between kenaf (**C**) lignin units is the γ-O-4 aryl ether which forms linear lignin chains; therefore, it is expected that the amount of monomers, released by β-aryl ether cleavage, from pine lignin will be lower than from kenaf lignin (see preceding paper). Fig. 2 clearly shows that the final degraded products by this method are those from lignin structural units linked through β-aryl ethers. In other words, the degraded monomer of pine lignin is 3-(4-acetoxy-3-methoxy)phenyl-propyl acetate from guaiacyl units linked by β-aryl ethers. Monomers from kenaf lignin are the same

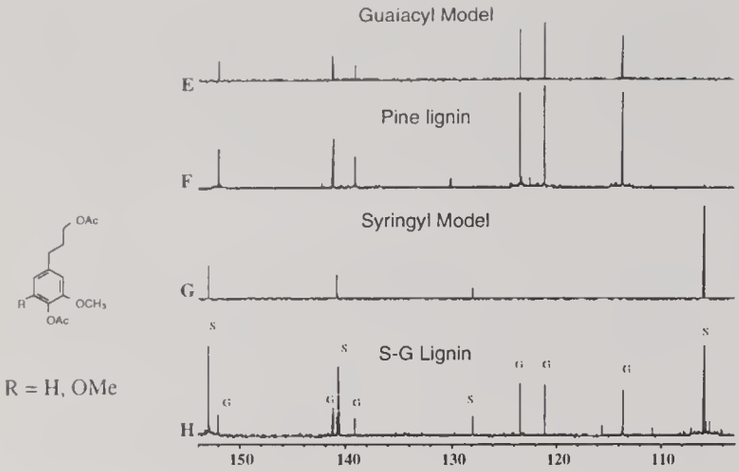


Figure 2. Sidechain region of ^{13}C NMR spectra of models and degradation products (mainly monomers) for lignins: E, guaiacyl model; F, pine lignin; G, syringyl model; H, an S/G lignin.

product from guaiacyl components as well as 3-(4-acetoxy-3,5-dimethoxy)phenylpropyl acetate from syringyl units connected by β-aryl ethers.

The new method for characterization of lignin provides us with the ability to look at both steps by NMR. After AcBr treatment, α-ethers are broken and the lignin has been derivatized but is otherwise intact (Fig. 1). Following β-ether cleavage in the two-electron reductive step, the lignin becomes extensively degraded. The material extracted into ether is primarily monomers with small amounts of higher oligomers.

The Advantage of Fully Digital NMR and Pulsed Field Gradients

J. Ralph

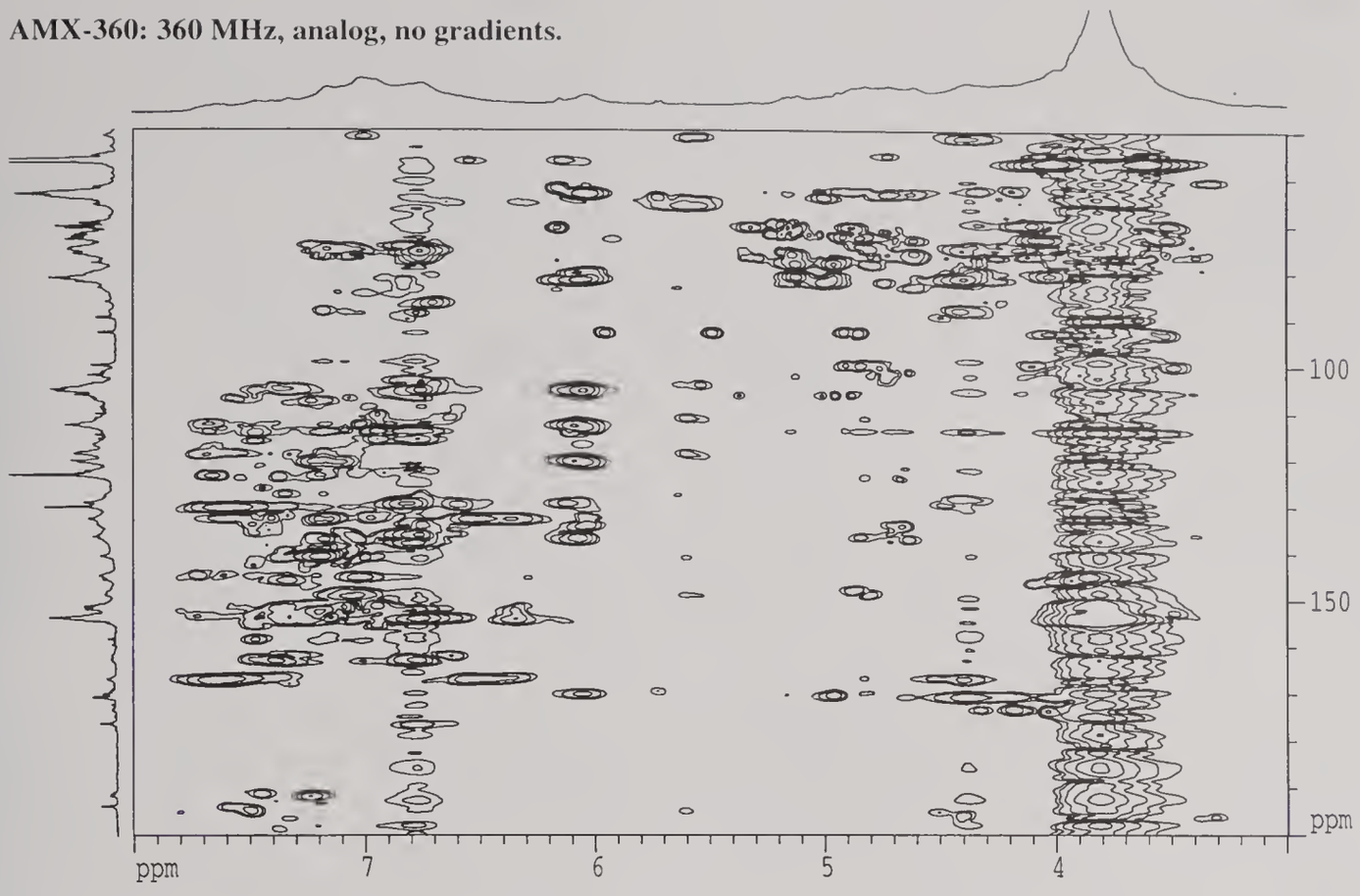
Introduction

NMR is the most revealing and diagnostic tool we have in cell wall structural research. Two significant advances have recently been made coupling advanced theory with new electronic and equipment designs.

The first is the ability to now make the entire NMR architecture digital. Up to this point, many of the steps involve analog signals with incipient limitations and constraints. Recent digital technology, now fully established and stable in commercial instrumentation, has allowed an enormous gain to be made over the now dated analog technology. In particular, absolutely square digital filtering, many orders of magnitude more sharply defined than was previously possible, has drastically reduced electronic artifacts and the folding in of signals and noise from outside the regions of interest. The consequent gain in sensitivity and selectivity (and the ability to perform a whole new class of NMR experiments) is substantial. It also provides spectra with absolutely flat baselines (or baseplanes in 2- and 3-dimensional experiments) improving the quantitative aspects and the ability to extract data from minor components near the noise level (for example, important cross-linking structures that are, without labeling under our current detectability level). Also, digital oversampling, via a technique similar to that used in CD players, extracts a further two-fold sensitivity gain. Note that the savings in time for an experiment goes as the square of the sensitivity ratio!

The second major advance is not as new but has not until quite recently been accessible to 'normal' NMR operations. That is the application of pulsed field gradients to NMR. The clever theoretical proposals for the benefits of applying huge, but finely controlled, ramped field gradients during an NMR experiment have now become so well implemented in modern NMR spectrometers that spectroscopists exploit them routinely. The results of the technology are spectacular. Because of the quantum mechanical properties of such pulses on nuclear spins, spectra acquired using gradients are virtually artifact free. Artifacts, particularly those produced by the dominant methoxyl groups in our samples, obscure and obfuscate the data of smaller components such as, for example, special cell wall cross-linking structures.

AMX-360: 360 MHz, analog, no gradients.



DMX-750: 750 MHz, digital, gradients.

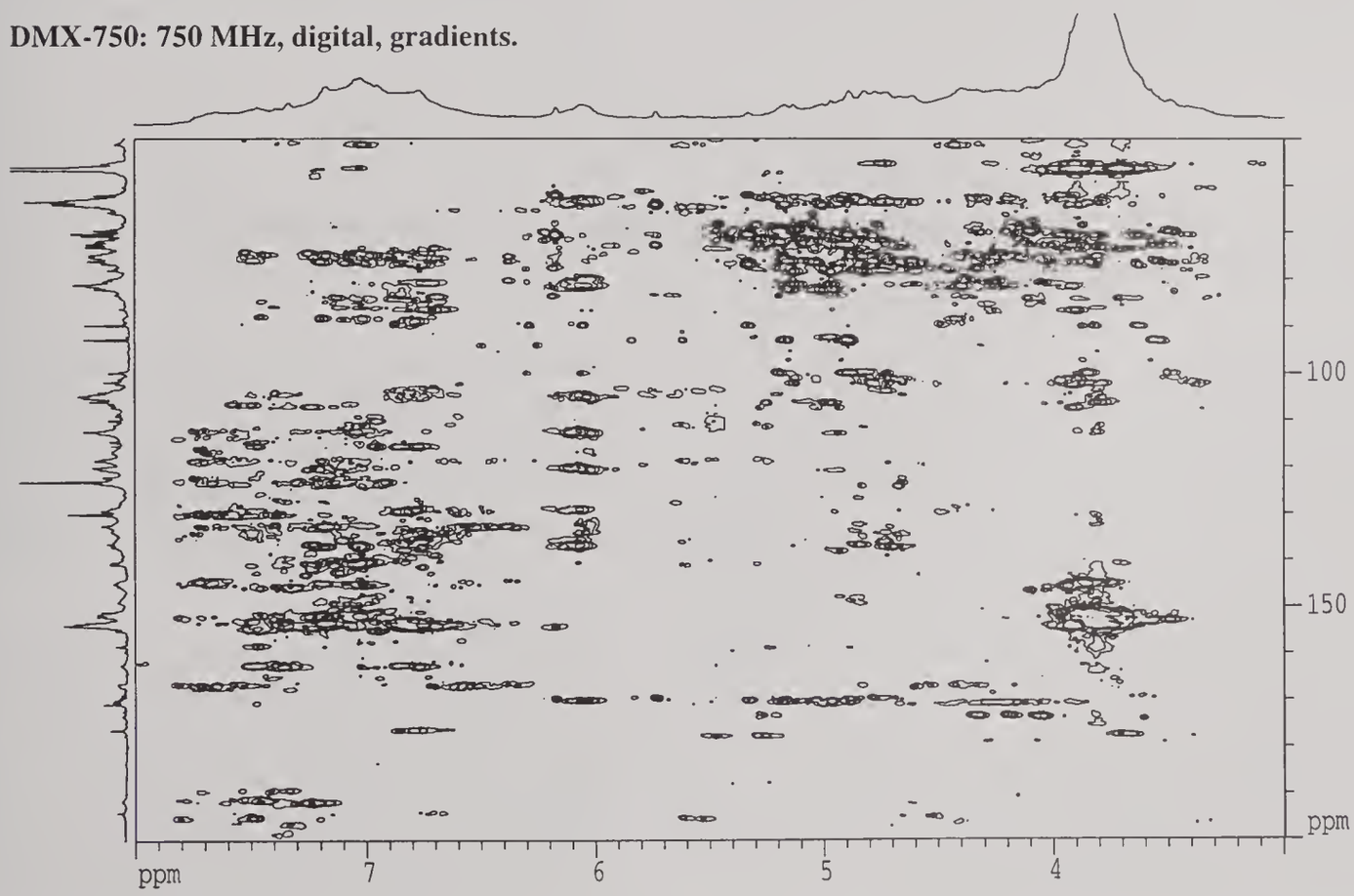


Figure 1. Comparison (unfair) of an HMBC spectrum of a ryegrass lignin with and without gradients and digital acquisition. Top spectrum: AMX-360, 300 mg, 60 h on our non-digital machine without gradients. Bottom spectrum: DMX-750, 122 mg, 24 h. Note particularly the absence of T_1-noise artifacts obfuscating the methoxyl region in the top spectrum (ca 4 ppm on the proton scale), the richness of detectable peaks due to the ability to approach the flat base-plane more closely (bottom spectrum), and the enhanced dispersion resulting from the MHz difference.

Methods

Long range ^{13}C—^{1}H correlation (HMBC) spectra of a uniformly ^{13}C-enriched ryegrass lignin were run on two NMR machines, our own AMX-360 and the National NMR Facilities DMX-750. On the 360, 300 mg of sample was dissolved in 0.24 ml of acetone-d_6 and run for 60 h to give the top spectrum in Fig. 1. On the 750 (which currently only has a 5 mm probe, 122 mg in 0.4 ml acetone-d_6 was run for 24 h to give the bottom spectrum.

Results and Discussion

The differences in the spectra, Fig. 1, are spectacular. Ignoring the obvious dispersion gain from the higher field instrument, the freedom from T_1-noise artifacts in the digital/gradient system (boom spectrum) is striking. Peaks close to the methoxyl region are no longer ambiguous. In addition, the flat baseplanes and improved sensitivity allow us to look closer down to the noise level to reveal many more potentially valuable correlations. The only drawback is that we now have a great many more assignments to make in this spectrum!!

Conclusions

The Center's NMR instrumentation, bought with support from our Area Office and each of the Center's Scientists, has been a tremendous asset to the Cell Wall program. The current instrument still runs non-stop, producing an array of valuable spectra from dozens of different NMR experiments. We have always been at the detectability limit, pushing the envelope of the technology to unravel the mysteries of the cell wall. What is exciting is that NMR has advanced significantly since the acquisition of our machine. The technological advances described above would still leave us close to the limit, but this data will become available on *unlabeled* (natural) materials and allow us to extend the observations to the other plants of interest (as well as further the investigations that are on-going). The large gain in sensitivity via the digital system and oversampling, the ability to detect much closer to the noise level because of absolutely flat baselines/planes, and the tremendous artifact reductions available using gradients makes the updated system a must-have in this type of research.

Evidence for the Role of Sinapyl *p*-Coumarate in Maize Lignification

J.H. Grabber, S. Quideau and J. Ralph

Introduction

Maize lignin contains up to 20% *p*-coumaric acid (**1**, Fig. 1) which is esterified at the γ-position of phenylpropane side-chains. Attachment at the γ-position indicates that *p*-coumaric acid is esterified intracellularly to *p*-hydroxycinnamyl alcohols (**2**); the resulting *p*-hydroxycinnamyl *p*-coumarates (**3**) are exported to the cell wall where they participate in oxidative coupling processes associated with lignification. This summary describes the successful use of thioacidolysis/desulfurization experiments to determine whether *p*-coumaroylated lignins in maize are formed primarily with coniferyl or sinapyl *p*-coumarate.

Methods

Thioacidolysis was performed on 10 mg of lignin, 40 mg of cell walls, and 2 to 5 mg of model compounds. Thioethylated products were hydrogenated with Raney nickel, trimethylsilyated, and analyzed by GC-MS and by GC-FID. The synthesis of model compounds has been previously reported (1989 and 1993 Research Summaries).

Results and Discussion

Ether linkages in maize lignin were cleaved by thioacidolysis to release syringyl and guaiacyl units acylated with *p*-coumaric acid. After hydrogenation with Raney nickel, GC-MS analysis of thioacidolysis products revealed two previously unidentified peaks with mass spectra consistent with compounds **6a** and **6b**, hydrogenated forms of coniferyl and sinapyl *p*-coumarate (Figs. 1 and 2). The identity of these peaks was confirmed by comparing their GC retention times and mass spectra with authentic compounds. Recovery of *p*-

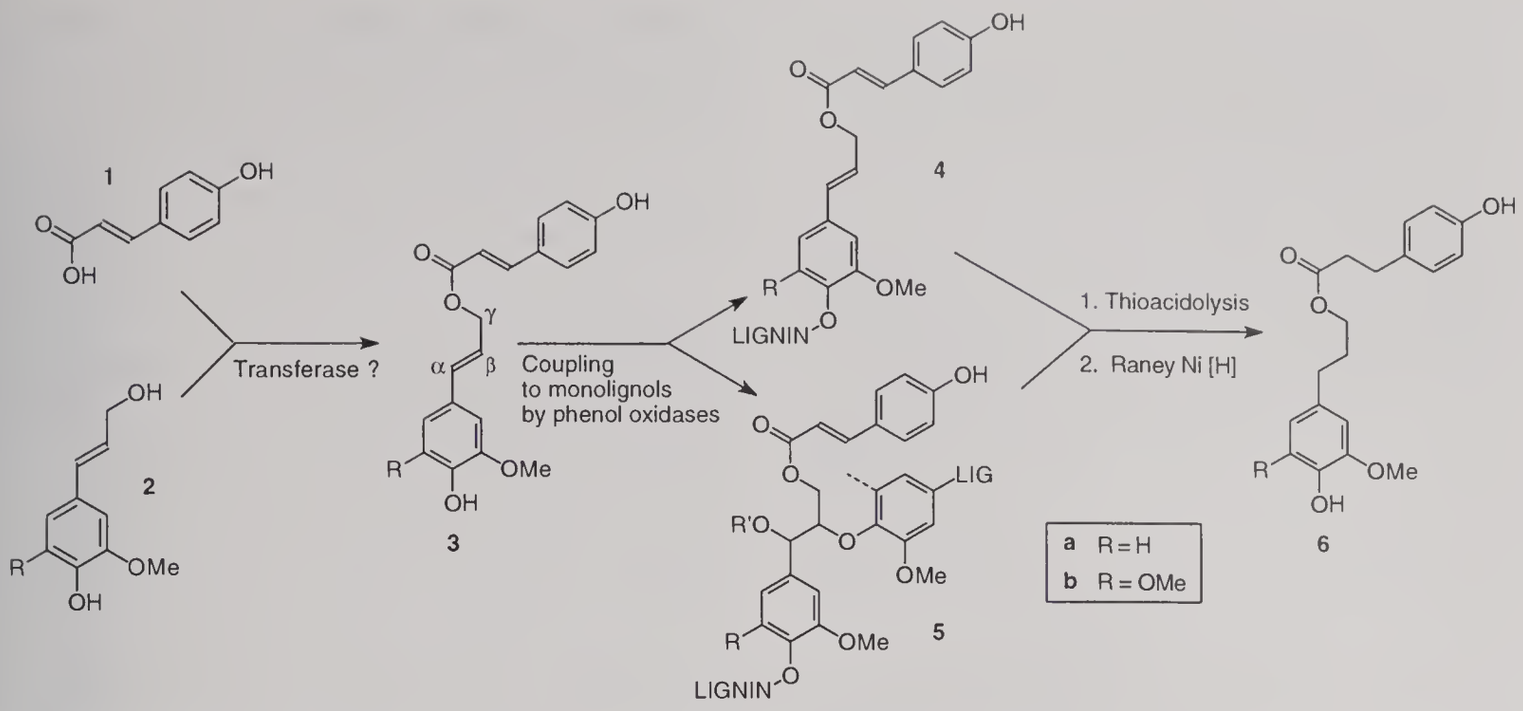


*Figure 1. p-Coumaric acid (**1**) esterification by p-hydroxycinnamyl alcohols (**2**) forms p-hydroxycinnamyl p-coumarates (**3**) which are incorporated into lignin to give, among other products, two types of β-ether structures: the hydroxycinnamyl p-coumarate end group **4** and the β-ether **5**. Thioacidolysis cleaves β-ethers while leaving a small proportion of the p-coumarate esters intact. After Raney nickel treatment, products **6a** and **6b** result, providing a distinction between coniferyl p-coumarate and sinapyl p-coumarate involvement in lignification.*

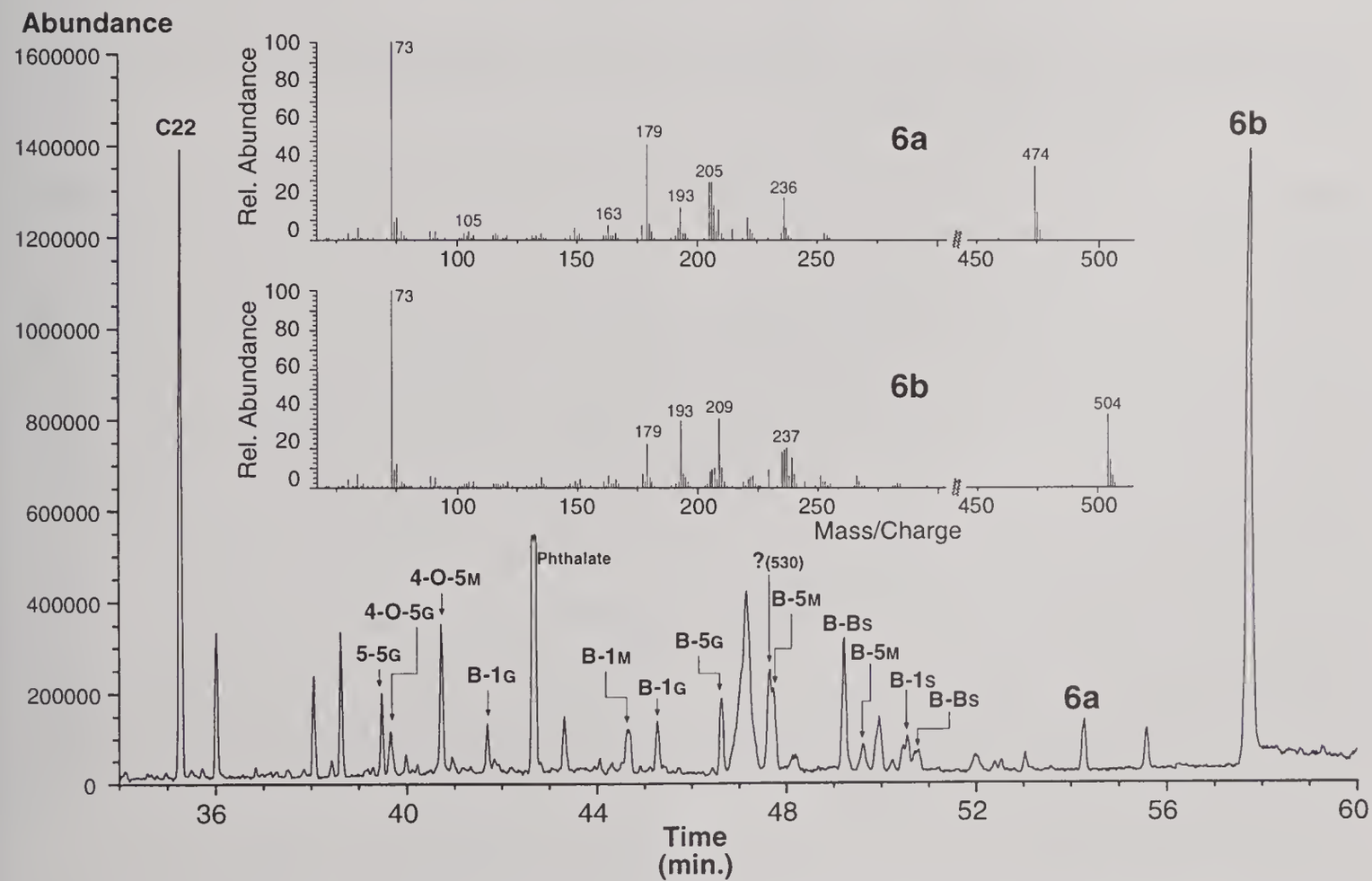


*Figure 2. GC-MS of dimers recovered after thioacidolysis of maize lignin. Dimers were trimethylsilyated prior to analysis. p-Coumarate esters (**6a** and **6b**) were observed later in the total ion chromatogram than previously identified syringyl-guaiacyl products.*

hydroxycinnamyl *p*-coumarates was low due to ester cleavage and incomplete β-ether cleavage by thioacidolysis. Yields were not improved when thioacidolysis was conducted under reduced duration or temperature. Hydrogenation of phenylpropane side chains with Pd/C prior to thioacidolysis also did not improve the recovery of *p*-coumarate esters. The possibility of sinapyl *p*-coumarate being an artifact formed during thioacidolysis was discounted by subjecting a mixture of willow lignin (a syringyl-guaiacyl lignin with no *p*-coumarate esters) and a model of β-ether **5a** to thioacidolysis/desulfurization. Although small quantities of coniferyl *p*-coumarate (**6a**) were recovered, sinapyl *p*-coumarate (**6b**) was not, confirming that sinapyl *p*-coumarate was a component of maize lignin and not an artifact formed by transesterification reactions. Peak areas of sinapyl *p*-coumarate (**6b**) were eight to nine times greater than that of coniferyl *p*-coumarate (**6a**), indicating that *p*-coumarate moieties were primarily attached to syringyl units. Since acylation should not differentially affect the release of syringyl or guaiacyl units from β-ether linked structures, our results strongly suggest that sinapyl *p*-coumarate (**3b**) was the major precursor of *p*-coumaroylated maize lignin.

Conclusions

Analysis of thioacidolysis/Raney nickel products has demonstrated that *p*-coumaric acid is esterified primarily by syringyl units in maize lignin, providing compelling evidence that sinapyl *p*-coumarate is the major precursor of *p*-coumaroylated lignins in grasses. Recovery of *p*-hydroxycinnamyl *p*-coumarates was low. Modifications of the analytical procedure did not significantly improve yields. Current work in our laboratory is focused on understanding the biosynthesis, transport, and oxidative coupling reactions of *p*-hydroxycinnamyl *p*-coumarates.

Severe Inhibition of Fiber Degradation by Cinnamyl Aldehyde-Containing Lignins

J.H. Grabber, J. Ralph and R.D. Hatfield

Introduction

Lignins are formed by peroxidase/H_2O_2-mediated polymerization of *p*-coumaryl, coniferyl and sinapyl alcohols. In plants where cinnamyl-alcohol dehydrogenase (CAD) activity is low (e.g. brown midrib mutants and CAD antisense plants), *p*-hydroxycinnamyl aldehydes probably also become a major component of lignin. It is not known whether lignin composition affects the degradability of cell wall polysaccharides. Such information would provide a rational basis for directing plant selection or molecular engineering efforts aimed at improving the bioconversion of structural polysaccharides into metabolizable energy for livestock or into ethanol fuels. We used a biomimetic model system to determine if alterations in lignin composition affect the degradability of cell walls.

Methods

Cell walls from cell suspensions (*Zea mays* L. cv. Black Mexican) were synthetically lignified with several types of monolignols. Cell walls were analyzed for neutral sugars, uronic acids and Klason lignin. Cell walls (100 mg in 10 mL of 20 mM acetate buffer, pH 4.8, 39°C) were degraded with hydrolases from *Trichoderma reesei* (4 µL of Celluclast, NOVO) and *Aspergillus niger* (4 µL of Viscozyme L, NOVO). Supernatant samples were analyzed for total sugars and uronic acids and for neutral sugars after TFA hydrolysis.

Results and Discussion

Nonlignified cell walls were rapidly and extensively degraded by fungal hydrolases (Table 1). Lignins formed with coniferyl aldehyde were much more inhibitory to cell wall degradation than lignins formed with coniferyl, sinapyl or *p*-coumaryl alcohols (Table 2 and 3). Cell-wall digestion by mixed rumen microorganisms was also severely restricted by aldehyde-containing lignins. Lignification, particularly with coniferyl

aldehyde, reduced the release of all neutral and acidic sugars by fungal hydrolases, especially that of xylose. When aldehyde groups were selectively reduced to alcohols (by ethanolic-sodium borohydride) prior to enzyme hydrolysis, degradability differences were largely eliminated. This suggests that high hydrophobicity of aldehyde-containing lignin was responsible for depressed cell wall degradation.

Conclusions

Our results indicate that incorporation of *p*-hydroxycinnamyl aldehydes into lignin, via down regulation of CAD, will severely restrict the bioconversion of structural polysaccharides into metabolizable energy for livestock and into ethanol fuels. In contrast, altering the type of *p*-hydroxycinnamyl alcohol incorporated into lignin is not expected to change the enzymatic degradation of plant cell walls.

Table 1. Lignin concentration and fungal-hydrolase degradability of cell walls synthetically lignified with coniferyl alcohol and coniferyl aldehyde.

Monolignol	Klason lignin	Total sugars released 6 h	72 h
	mg/g of cell wall		
None (nonlignified)	6[a]*	421[a]	750[a]
Coniferyl alcohol	133[b]	201[b]	487[b]
Coniferyl aldehyde	141[b]	104[c]	306[c]
Coniferyl alcohol + coniferyl aldehyde (1:1 ratio)	139[b]	136[c]	400[bc]
CV%	8.7	9.0	8.4

*Means followed by the same letter are not significantly different ($P < 0.05$).

Table 2. Lignin concentration and fungal-hydrolase degradability of cell walls synthetically lignified with *p*-coumaryl, coniferyl and sinapyl alcohols.

Monolignol	Klason lignin	Total sugars released 6 h	72 h
	mg/g of cell wall		
Coniferyl alcohol	111[a]	221[a]	530[a]
Coniferyl + *p*-coumaryl alcohol (1:1 ratio)	111[a]	212[a]	507[a]
Coniferyl + sinapyl alcohol (1:1 ratio)	102[a]	220[a]	527[a]
CV%	5.2	5.6	7.1

*Means followed by the same letter are not significantly different ($P < 0.05$).

Ferulate Polysaccharide Esters as Nucleation Sites for Lignification

J.H. Grabber and J. Ralph

Introduction

Biomimetic lignification systems and polysaccharide-lignin isolates from grasses have revealed that ferulate polysaccharide esters are oxidatively coupled to lignin. Model studies with maize cell suspensions were conducted to determine if ferulates act as nucleation sites for lignification and to provide detailed information on the types of cross-products formed with ferulate esters and coniferyl alcohol.

Methods

Cell walls isolated from maize cell suspensions were treated with dilute hydrogen peroxide and synthetically lignified with 0 to 8 equilavents of coniferyl alcohol per unit of cell wall ferulate. Cell walls with γ–^{13}C labelled ferulate were treated with dilute hydrogen peroxide and lignified with one-equilavent of coniferyl alcohol or β-5 dehydrodiconiferyl alcohol per unit of wall ferulate. Cell walls were subjected to alkaline hydrolysis at room temperature or at 170°C to release ferulates and cross-products formed between ferulates and lignin. Hydrolysates were acidified and extracted with ethyl acetate. Extracts were analyzed by GC-FID, GC-MS, and NMR.

Results and Discussion

Nonlignified walls were incubated with an excess of H_2O_2 to stimulate oxidative coupling of ferulate monomers into dehydrodimers by wall-bound peroxidase. Dehydrodimers comprised 55% of total

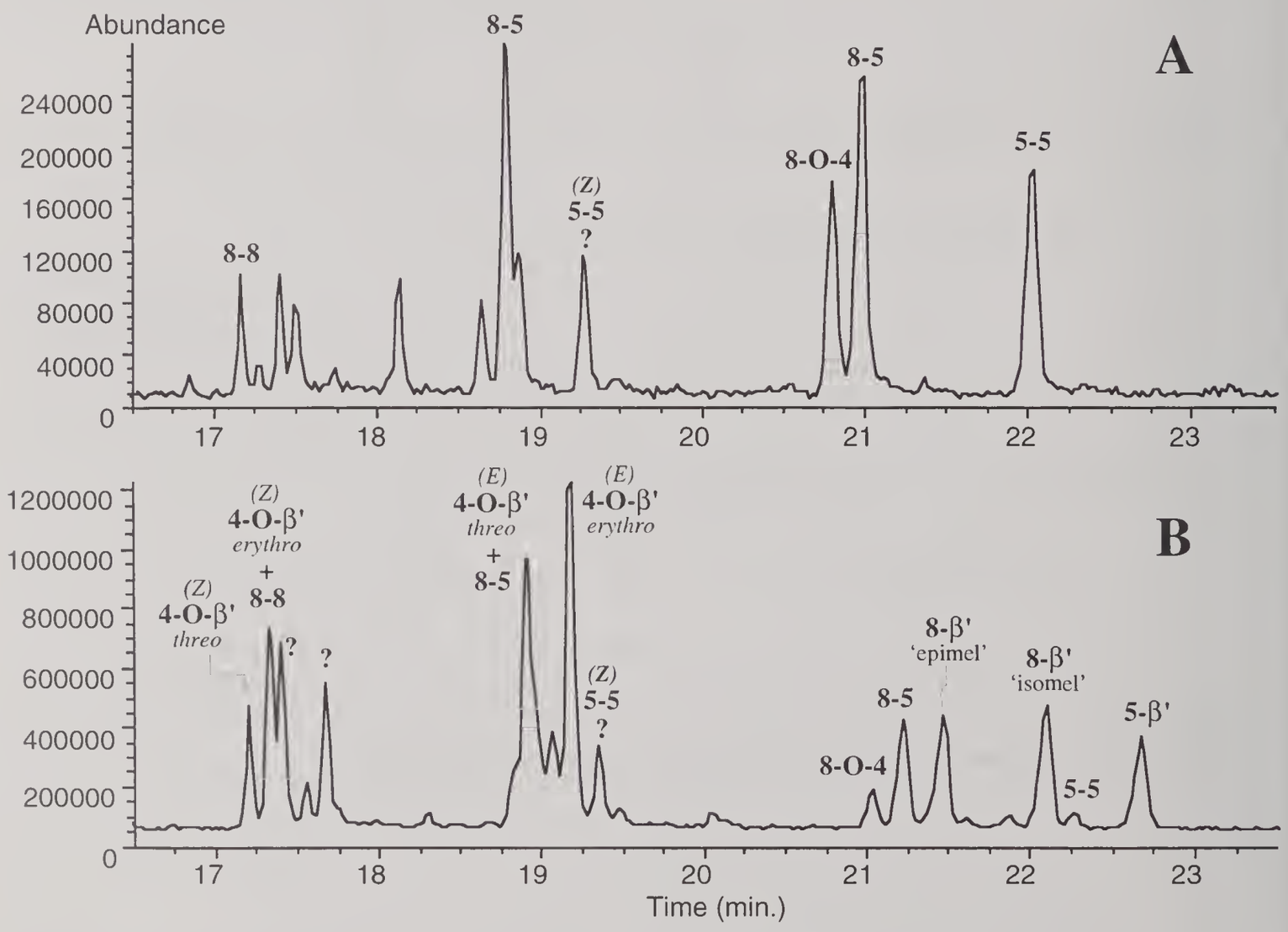


Figure 1. GC-MS total ion chromatogram of ferulate and ferulate-coniferyl alcohol cross-products recovered after saponification of (a) nonlignified maize walls and (b) maize walls lignified with coniferyl alcohol.

Figure 2. C-H correlation (HMBC) spectra of ferulates and ferulate-lignin cross-products recovered after saponification of (a) nonlignified maize walls and (b) maize walls lignified with coniferyl alcohol. Folded-in peaks are grayed. Correlations corresponding to a new product are currently under investigation.

ferulates in H_2O_2-treated walls. This high degree of dimerization is remarkable considering the low concentration of ferulates (17 mg/g) in cell walls. Ferulate moieties of dehydrodimers were coupled primarily by 8-5 linkages (53%) followed by 8-O-4 (20%), 5-5 (17%) and 8-8 (10%) linkages. Ferulate monomers and 5-5 coupled dehydrodimers were extensively incorporated into lignin (95%). In contrast, dehydrodimers coupled at the 8-position had a lower propensity to incorporate into lignin (78 to 90%). The incorporation of dehydrodimers into lignin is probably controlled by steric factors and by the number of phenols available for radical coupling reactions.

GC-MS and NMR analyses of wall extracts and authentic compounds revealed ferulate-coniferyl alcohol dehydrodimers were coupled exclusively by 4-O-β', 8-β', and 5-β' linkages (Fig. 1). (In labeling cross-products, the first term indicates the coupling site on the ferulate ester, and the primed term indicates the coupling site on the lignin moiety.) The absence of 8-5', 8-O-4' and 5-5' coupled cross-products indicates that ferulates couple exculsively to the β–position of coniferyl alcohol monomers; coupling at other positions occurs only with coniferyl alcohol dimers and oligomers in which the conjugated side chain is no longer present. Ferulate monomers and dehydrodimers differed substantially in their propensity to form ether-linked structures with lignin; high temperature alkaline hydrolysis released 30% of ferulate monomers compared to 28% of 5-5, 41% of 8-8, 61% of 8-O-4, and *ca* 60% of 8-5 coupled dehydrodimers.

Conclusions

At the initiation of lignification, arabinoxylans become extensively cross-linked by oxidative coupling of ferulate monomers into dehydrodimers. Ferulate monomers and dehydrodimers act as nucleation sites for lignification and their incorporation into lignin results in further cross-linking of the cell wall matrix. Ether linkages comprised 30 to 60% of the linkages between ferulates and coniferyl alcohol.

Effect of Lignin Composition on Cell-Wall Degradability

H.G. Jung and C.C.S. Chapple

Introduction

Lignification of forage cell walls is regarded as the primary mechanism whereby ruminal cell-wall degradability is inhibited. The USDFRC Cell Wall Group has identified ferulic acid cross-linking of lignin to polysaccharide in grass cell walls as an important modifier of the negative effects of lignin on cell-wall degradability. Another hypothesis which has been proposed to explain observed variation in the effect of lignin on cell-wall degradability regards the composition of lignin. Most lignin in forage crops is derived from coniferyl and sinapyl alcohol monolignols. These monolignols differ only in the presence of an extra methoxyl group on the aromatic ring of sinapyl alcohol compared to coniferyl alcohol. This extra methoxyl will prevent bonding of sinapyl monolignols at the C-5 position which should result in a more linear, less highly branched lignin resulting from predominantly sinapyl monolignols. The brown midrib (bmr) mutants of annual C_4 grasses such as maize have defects in lignin biosynthesis such that their lignin is richer in coniferyl alcohol monolignols. These bmr mutants also have cell walls which are more extensively degraded than the normal type. As a result, it has been proposed that a shift from a typical mixed coniferyl/sinapyl alcohol derived lignin to lignins richer in coniferyl alcohol units should alter cell-wall degradability. The identification of a genetic mutant in *Arabidopsis thaliana* that is incapable of producing any sinapyl alcohol affords the opportunity to test the most extreme manifestation of this hypothesis concerning the effect of lignin composition of cell-wall degradability.

Materials and Methods

Wild-type and mutant *Arabidopsis* plants, back-crossed to the wild-type parent for either 2 (*fah*1-2) or 5 (*fah*1-5) generations, were grown in the greenhouse. After initiation of flower stalk development, plants were harvested at weekly intervals to yield stem samples of four different maturities. Approximately 450 plants of each genotype were harvested at each maturity stage. Two replications of the experiment were conducted. The stems were lyophilized, ground to pass a 1-mm screen in a cyclone mill, and analyzed for cell-wall composition. In vitro degradability of cell-wall polysaccharides was determined after 24- and 96-h incubations with rumen fluid.

Results and Discussion

Previous research using nitrobenzene oxidation and pyrolysis-GC-MS demonstrated that the mutant *Arabidopsis* plants produce no sinapyl alcohol derived lignin. While cell-wall concentration and lignin concentration in the cell wall changed with maturity (Fig. 1), virtually no differences in cell-wall composition among the *Arabidopsis* genotypes were observed (Table 1). It should be noted that the cell-wall composition of *Arabidopsis* is very similar to legumes such as alfalfa. The only significant effect of the mutation preventing sinapyl alcohol biosynthesis was an increased concentration of ferulic acid esters in the mutant lines. Degradability of the cell-wall polysaccharides did not differ among genetic lines. As expected, degradability declined with maturity, but there was no increase in degradation associated with the longer fermentation interval (Fig. 2). This indicates that those cell-wall polysaccharides which are susceptible to degradation are degraded very rapidly. This is similar to the pattern for cell-wall degradability seen in legume forages.

Conclusion

While *Arabidopsis* will never be a forage crop for dairy cattle, its cell-wall composition suggests that this species can serve as a model for legumes. Our results indicate very clearly that when lignin concentrations are the same, as was the case for these *Arabidopsis* lines, lignin composition does not impact cell-wall polysaccharide degradation. These results also imply that the effect of the bmr mutation in maize and other grasses on cell-wall degradability is probably a result of reduced cell-wall development and lignin concentration rather than the alteration in lignin composition.

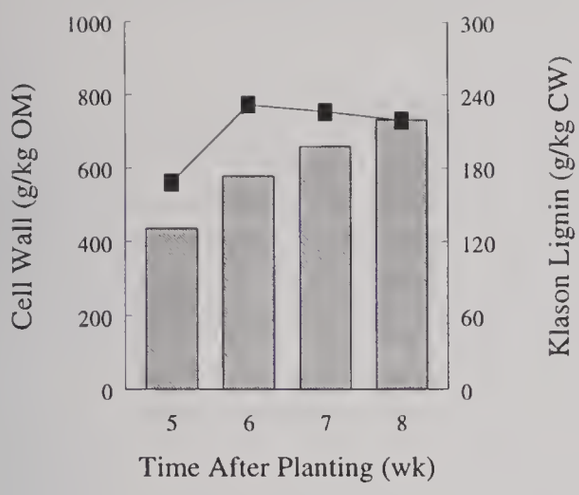


Figure 1. Change in cell-wall concentration (bar) and lignification (line) of Arabidopsis stems harvested at different stages of development, averaged across the three genetic lines.

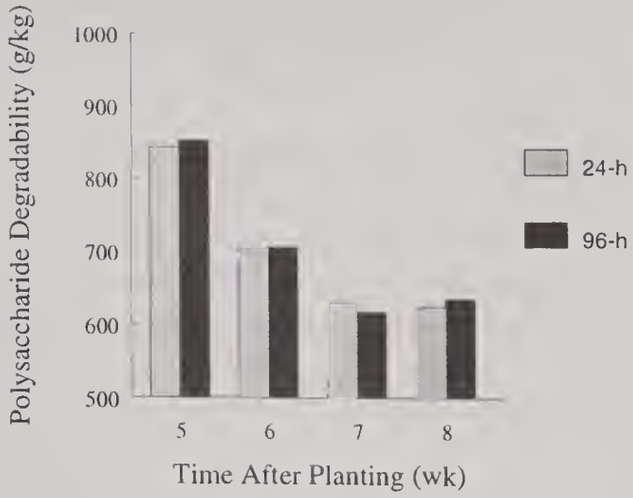


Figure 2. In vitro ruminal cell-wall polysaccharide degradability of Arabidopsis stems harvested at different stages of development after 24- and 96-h fermentations, averaged across the three genetic lines.

Table 1. Stem cell-wall composition of *Arabidopsis* lines averaged across maturity stages.

	Genetic line			
Cell-Wall Trait	Wild-type	*fah*1-2	*fah*1-5	SEM
Cell wall, g kg^{-1} OM	589	615	600	14
Composition, g kg^{-1} CW				
Neutral sugars	577	574	573	7
Uronic acids	207	205	207	3
Klason lignin	208	212	213	7
Ferulic acid				
esters	0.50[a]	0.82[b]	0.94[b]	.10
ethers	4.94	5.54	5.01	.43
p-Coumaric acid				
esters	0.48	0.55	0.37	.08
ethers	1.87	1.13	1.24	.29
Molar proportions of neutral sugars, mol 100 mol^{-1}				
Glucose	57.3	57.5	57.6	.3
Xylose	21.6	21.8	21.1	.3
Arabinose	4.8	4.7	4.9	.1
Galactose	6.9	6.9	7.2	.2
Mannose	5.8	5.9	6.1	.1
Rhamnose	3.1	2.8	2.9	.1
Fucose	0.43	0.42	0.35	.04

[ab]Means in the same row not sharing a superscript are different ($P < 0.05$).

Alterations in Cell-Wall Composition of Transgenic Alfalfa Due to Expression of a Fungal Mn-Dependent Peroxidase Gene

H.G. Jung, S. Austin-Phillips and D.R. Mertens

Introduction

Biochemical pulping of wood is desired as a method to reduce the environmental pollution associated with current chemical pulping technologies. Lack of supply and production costs of peroxidase enzymes, enzymes capable of both polymerizing and de-polymerizing lignin, needed in biochemical pulping is a major road-block to its development. Plants have been suggested as a vehicle to produce large amounts of industrial enzymes such as peroxidase through application of biotechnology. A Mn-dependent peroxidase from *Phanerochaete chrysosporium*, a lignin degrading aerobic fungus, has been successfully transferred into alfalfa. These transgenic plants are capable of producing the peroxidase; however, abnormal growth characteristics are observed in those plants which are high expressors of the peroxidase protein. The transgene protein product was targeted to be secreted out of the cell to the apoplast. Electron microscopy confirmed the presence of the enzyme in the apoplast and in the cell walls. We have examined the cell-wall composition of these transgenic plants for alterations induced by production of this fungal peroxidase.

Materials and Methods

Vegetatively propagated plants from eight alfalfa transformants were grown in the field in a replicated trial. Based on a Southern analysis, four of the transformants had zero, one or two copies of the transgene but did not produce the peroxidase protein product. Three of the other transformants had one copy of the transgene and one transformant had an unknown number of transgene copies. These latter four transformants all produced high levels of the peroxidase enzyme product of the transgene. The plants were harvested at flowering, separated into leaf and stem fractions, lyophilized, and ground to pass a 1-mm screen in a cyclone mill. Samples were analyzed for cell-wall concentration and composition (neutral sugars, uronic acids, Klason lignin, and esterified and etherified *p*-coumaric and ferulic acids).

Results and Discussion

The expression of the fungal Mn-dependent peroxidase had significant effects on the cell-wall concentration and composition of transgenic alfalfa plants (Table 1). Interestingly, leaves and stems responded differently to the presence of the peroxidase transgene product. Cell-wall concentration increased in leaves of plants expressing high levels of the transgene whereas the opposite effect was seen in the stems. Uronic acid content of the cell walls was increased in stems, but decreased in leaves, by expression of the transgene. Because peroxidases are needed for the polymerization of lignin during its biosynthesis, it was expected that lignin concentration would be impacted by the transgene. For leaves, an increase in Klason lignin content of the cell wall was observed for the high expressor plants, but no alteration occurred in high expressor stem tissue. The only consistent responses for both leaves and stems to the presence of the transgenic peroxidase were increases in ferulic acid esters in the cell wall and the molar proportion of arabinose among the neutral sugars. The increase in arabinose proportion was at the expense of glucose in the leaves, but in stems xylose was the neutral sugar which declined.

Conclusion

The presence of extra peroxidase in the apoplast and cell wall due to introduction of a transgene might be expected to alter cell-wall composition and structure because peroxidase is involved in cell-wall lignification and cross-linking. Why leaves and stems of the transgene expressing alfalfa plants reacted in opposite directions to the same genetic modification is not clear. Obviously the tightly controlled development of plant cell walls is extremely sensitive to manipulation and reacts in unexpected ways. The apoplast of plants may not be a suitable deposition site for transgenic production of reactive industrial enzymes.

Table 1. Cell-wall concentration and composition of transgenic alfalfa plants which were negative or high expressors of a fungal Mn-dependent peroxidase transgene.

	Leaves		Stems	
Trait	Negative	High	Negative	High
Concentration, g kg^{-1} OM				
Cell wall	254	313*	666	609*
Composition, g kg^{-1} CW				
Neutral sugars	501	474	632	617
Uronic acids	305	254*	134	143*
Klason lignin	191	270*	234	239
Esters				
p-Coumarate	0.31	0.27*	0.09	0.09
Ferulate	0.40	0.48*	0.10	0.13*
Ethers				
p-Coumarate	0.53	0.44*	0.13	0.13
Ferulate	0.44	0.52	0.27	0.29
Neutral Sugar Composition, mol 100 mol^{-1}				
Glucose	56.2	44.8*	56.8	56.9
Xylose	4.9	7.6	29.5	27.1*
Arabinose	19.3	26.1*	7.2	8.7*
Galactose	13.7	15.3	4.0	4.7
Mannose	6.3	6.3	2.4	2.7

*High expressor transformants are different ($P < 0.10$) from negative expressors, within a plant part.

Screening for Cell-Wall Mutants Deficient in Ferulate Ester Synthesis in Maize

W. Ni, R.L. Phillips and H.G. Jung

Introduction

Ferulic acid (FA) esters are involved in the cross-linking of lignin to polysaccharides in plant cell walls. Such cross-linking exerts a great impact on cell-wall accessibility, extensibility, and plasticity and also adversely affects cell-wall digestibility. Reduction of this cross-linking has markedly increased the rate and extent of cell-wall digestibility in a maize cell culture system. The goal of our research is to develop maize lines with increased cell-wall digestibility for use as silage for the feeding of dairy and beef cattle. We intend to identify and clone the gene responsible for FA ester synthesis and the gene will be reversely engineered back into plants to reduce the cross-linking. Our approach is to screen transposon tagged maize lines for mutants with reduced FA ester concentration in order to identify and isolate the gene of interest.

Materials and Methods

The transposon tagged maize lines were obtained by crossing the mum-9 transposon mutator into four inbred maize lines (A188, A619, A632, and W23). The selfed F_2 and F_3 progeny from these crosses were grown either in the greenhouse or in the field. The first true leaves were removed from 14 to 21 d old seedlings and dried at 50°C overnight. The samples were weighed and incubated in 1 ml of 2 N NaOH (20 h at 39°C) to release phenolic esters. The extracts were acidified with 6 N HCl to pH 1.5-2, placed on ice for 1 hr, and centrifuged for 10 min. The supernatant was transferred to sample vials for phenolic analysis using HPLC. Ferulic and *p*-coumaric (PCA) acid concentrations were estimated using commercial FA and PCA as external standards. Plants that had FA concentrations or FA/PCA ratios two standard deviations above or below

the line mean were selected for further analysis and selfed. Mature tissues from selected plants were analyzed using the same procedure for their FA and PCA ester concentrations.

Results and Discussion

More than 12,000 maize seedlings have been analyzed for their FA and PCA ester concentrations and 397 plants were identified with unusually low or high FA concentrations and FA/PCA ratios. Of the selected plants, 140 produced viable seed. After analyzing mature tissues of the 140 selected seed-bearing plants, only 10 plants exhibited the desired traits of low FA concentrations or low FA/PCA ratios as both seedlings and at maturity. The progeny of these 10 plants have been evaluated in a greenhouse trial to verify the heritability of the desired traits. Progeny of three plants passed this evaluation. Lignin composition analysis by pyrolysis-GC-MS analysis indicated that one of the three selected plants exhibits unusually low syringyl unit abundance.

During the 1995 growing season we sowed 12,000 seeds, but only 8300 (72%) germinated and survived to the initial sampling. To our surprise, none of the plants analyzed was deficient in ferulate esters. In fact, FA concentrations in the cell wall were maintained at fairly constant levels within populations of uniform genetic background (Fig 1). Distribution of FA concentrations and FA/PCA ratios were truncated at the low ends of the curve (Fig. 2), suggesting that plants with low FA concentrations or low FA/PCA ratios died prior to screening.

Table 1 summarizes the viability of the selected 397 plants. One hundred sixteen (29%) died before pollination. Most of these dead plants had either low FA concentrations or low FA/PCA ratios. Mortality for the entire nursery population was only 12%. One hundred twenty (30%) of the selected plants exhibited abnormal developmental and growth patterns while only 15% of the overall population showed such abnormalities. These observations suggest that a threshold FA concentration in the cell wall is needed to maintain normal plant growth and development.

Conclusion

Massive screening has produced three putative mutants with reduced FA ester synthesis. Gene cloning using a polymerase-chain-reaction based screening technique is in progress. While our goal continues to be improvement of cell-wall digestibility through reduced cross-linking, the data from our screening experiment suggest that complete removal of FA ester synthesis may be catastrophic to plant growth and development.

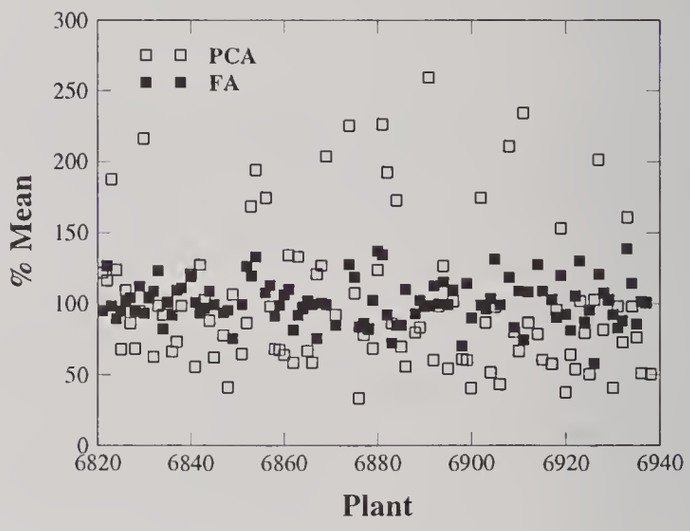


Figure 1. Distributions of FA and PCA concentrations. All 116 plants were from the same ear. Concentrations are expressed as a percent of the population mean.

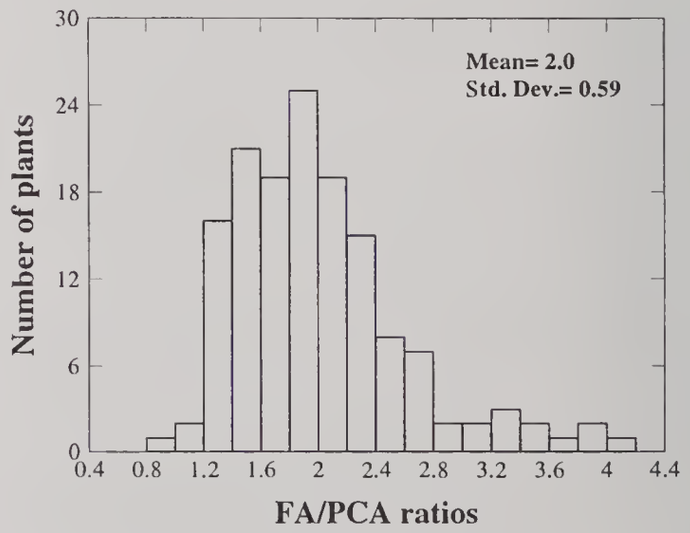


Figure 2. Distribution of FA/PCA ratios. All plants were from the same ear.

Table 1. Viability at maturity of maize plants selected based on seedling screening.

	High FA/PCA	Low FA/PCA	High FA	Low FA
Number of plants selected	130	162	19	87
Healthy plants	52	41	4	34
Dead plants	12	84	0	20
Abnormal	21	10	10	12
Male sterile	27	14	2	7
Female sterile	7	6	1	4
Infected Plants	1	7	2	10

Lignin Analysis of Resistant and Susceptible Aspen Trees Wounded and Inoculated with *Hypoxylon mammatum*, the Causal Agent of *Hypoxylon* Canker

B. Bucciarelli, C.P. Vance and H.G. Jung

Introduction

Aspen (*Populus tremuloides* Michx.), the predominant forest tree in the Great Lakes States, is widely grown for use in the paper and construction industries. *Hypoxylon mammatum* (Wahl.) Mill, the causal agent of Hypoxylon canker, is the most important pathogen of aspen. Unrestricted infection to the main bole of the tree results in loss of wood quality and increases the risk of tree breakage. Studies of adult trees show tremendous clonal variation towards susceptibility to *Hypoxylon* infection. The goal of this research was to identify characteristics that contribute to disease resistance or susceptibility. Previous studies have shown that both resistant and susceptible genotypes develop a lignified zone along the wound margin in response to either wounding only or wounding plus inoculation with the pathogen. The focus of this study was to determine if differences exist in lignin concentration and/or in the monomeric composition of the response lignin deposited by these two genotypes.

Materials And Methods

Green internodal stem tissue of greenhouse grown aspen was either wounded only or wounded plus inoculated with mycelium of *Hypoxylon mammatum*. Stem tissue (3 to 4 mm diameter) was sampled at 24-, 48-, 72- and 96-h after treatment. Lignin content of treated internodes and nonwounded controls was measured using the Klason lignin method. This method measures the lignin residue after the tissue is hydrolyzed in concentrated sulfuric acid. Pyrolysis-GC-MS was used to qualitatively analyze the lignin composition of stem tissue 96 h after treatment and in nonwounded controls.

Results

Klason lignin concentration of stem internodes was greater in the resistant aspen genotype in nonwounded controls (Fig. 1). After wounding only, both genotypes exhibited rapid increases in lignin concentration; however, the resistant genotype continued to consistently contain more lignin than the susceptible genotype. After wounding plus inoculation, both genotypes again rapidly deposited lignin, but in this treatment the susceptible aspen genotype had similar lignin concentrations to the resistant genotype at 24-, 48- and 72-h after inoculation. By 96-h after inoculation, the susceptible genotype actually contained more lignin than the resistant aspen.

Pyrograms from pyrolysis-GC-MS analysis showed that the syringyl-to-guaiacyl (S/G) ratio of monolignols for nonwounded controls was statistically similar for both resistant and susceptible genotypes (Table 1), suggesting that the initial syringyl and guaiacyl content of lignin for both genotypes are proportionately the same. Subsequently, in response to wounding only and wounding plus inoculation, there was a decrease in

the S/G ratio for both genotypes (Table 1). The decrease, however, was more pronounced for the susceptible than for the resistant genotype.

Analysis of the pyrograms indicated the presence of more hydroxyphenyl-type lignin in wounded only and wounded plus inoculated stem tissue from both genotypes than seen in nonwounded controls. This observation is based on the appearance of *t*-4-propenylphenol after wounding (Table 1). Additionally, the susceptible genotype contained more 4-vinylphenol after wounding only and wounding plus inoculation than in its nonwounded control or any of the resistant genotype samples. 4-Vinylphenol can arise during pyrolysis from hydroxyphenyl lignin and, also, from *p*-coumaric acid. Whether the increased 4-vinylphenol observed in the susceptible genotype due to wounding is from hydroxyphenyl lignin or *p*-coumaric acid is unknown.

Conclusion

The results indicate that lignin concentration of aspen increases in response to *H. mammatum* infection, but the susceptible and resistant genotypes are similar in the magnitude of this lignification response. However, the two genotypes display differences in the composition of the lignin polymer deposited. Both genotypes deposit a lignin lower in syringyl units in response to wounding, but additionally the susceptible genotype response lignin is enriched in hydroxyphenyl lignin and, perhaps, *p*-coumaric acid.

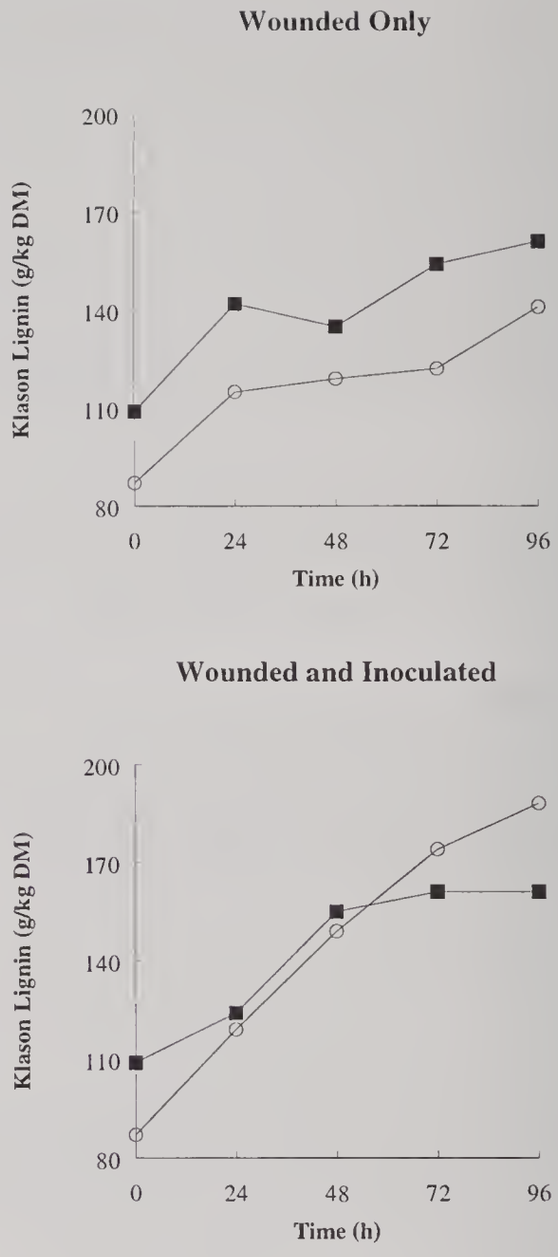


Figure 1. Klason lignin concentration of green internodal stem tissue of putative resistant (■) and susceptible (O) aspen that was wounded only or wounded and inoculated with Hypoxylon mammatum.

Table 1. Lignin composition of green internodal tissue from putative resistant and susceptible aspen 96-h after wounding only or wounding and inoculation with *Hypoxylon mammatum*.

Genotype-treatment	S/G Ratio	*t*-4-Propenylphenol	4-Vinylphenol
	- - - - - - - standardized to guaiacol - - - - - - -		
Susceptible-nonwounded	1.23	0	1.44
Susceptible-wounded	0.86	0.27	3.43
Susceptible-wounded & inoculated	0.93	0.22	3.18
Resistant-nonwounded	1.10	0.26	1.21
Resistant-wounded	0.93	0.19	1.50
Resistant-wounded & inoculated	0.99	0.19	1.50

Characteristics of Cell Wall Polysaccharides Isolated From Different Cell Types of Grain Sorghum

R.D. Hatfield and J. Wilson

Introduction

The high levels of potential energy in cell walls of forages are under utilized by ruminants, especially those requiring high energy inputs such as dairy cows. For grasses this poor utilization is partially due to the cross-linking of the wall components, particularly the attachment of polysaccharides to lignin. Model studies have shown that degradation of wall polysaccharides are not uniformly affected by increasing levels of lignin. Arabinoxylans are more resistant to degradation and those that are degraded tend to end up as oligosaccharides. We were interested in the degradation characteristics of different cell types isolated from grain sorghum, particularly why pith cell walls were apparently more resistant to degradation than vascular bundle cells or sclerenchyma cells (Wilson et al. 1993). This work was undertaken to evaluate differences in the types and quantity of structural polysaccharides isolated from walls of different sorghum stem cell types.

Material and Methods

Cell walls were isolated, using a modified Theander procedure, from different cell types separated from the fifth stem internode of plants at anthesis. Walls were subjected to a sequential chemical fractionation consisting of ammonium oxalate, delignification with sodium chlorite, hot water extraction, and stepwise increases in KOH (0.25M, 0.5M, 1.0M to 4.0M). Isolated fractions were analyzed for neutral sugar composition following 2N TFA hydrolysis.

Results and Discussion

Fractionation of the different cell wall types from sorghum stems into principal polysaccharide groups did not differ appreciably from each other even though they have different structural and functional roles. Table 1 shows that all of the different cell wall types resulted in similar amounts of total carbohydrate in each of the main groups of structural polysaccharides (pectic and hemicellulosic polysaccharides and cellulose residue). Pectic polysaccharides are defined here as all polysaccharides solubilized by ammonium oxalate, then sodium chlorite delignification, and lastly by a hot water extract. Hemicellulosics are those polysaccharides solubilized by KOH treatment, and the cellulose residue is what remains.

Neutral sugar composition of the different fractions varied little among the different cell wall types (Figs. 1 and 2). Delignification of walls typically solubilizes additional pectic type polysaccharides in legumes such as alfalfa; however, in these grass walls the major polysaccharide released would belong to the xylan group (Fig. 1). Pith cells have a wider distribution of neutral sugars in most of the polysaccharide fractions. This is most likely due to the lack of extensive secondary wall thickening that is seen in the other three types of cells, leaving the primary wall as a larger proportion of the total wall structure. The other cell wall types are more homogeneous in terms of sugar composition, suggesting that the secondary wall thickening of these cells are all similar.

Conclusion

Although the cell walls surrounding the different sorghum cell types appear anatomically different, polysaccharide composition appears to be quite similar. Degradation differences among these cell walls, particularly the less digestible pith walls, would not seem to be attributable to significant differences in structural polysaccharides. Instead it suggests that differences in pith wall digestibility may be due to increased cross-links among the different wall components.

Reference

Wilson, J.R, D.R. Mertens and R.D. Hatfield. 1993. Digestion, cell wall and anatomical characteristics. 63:407-417.

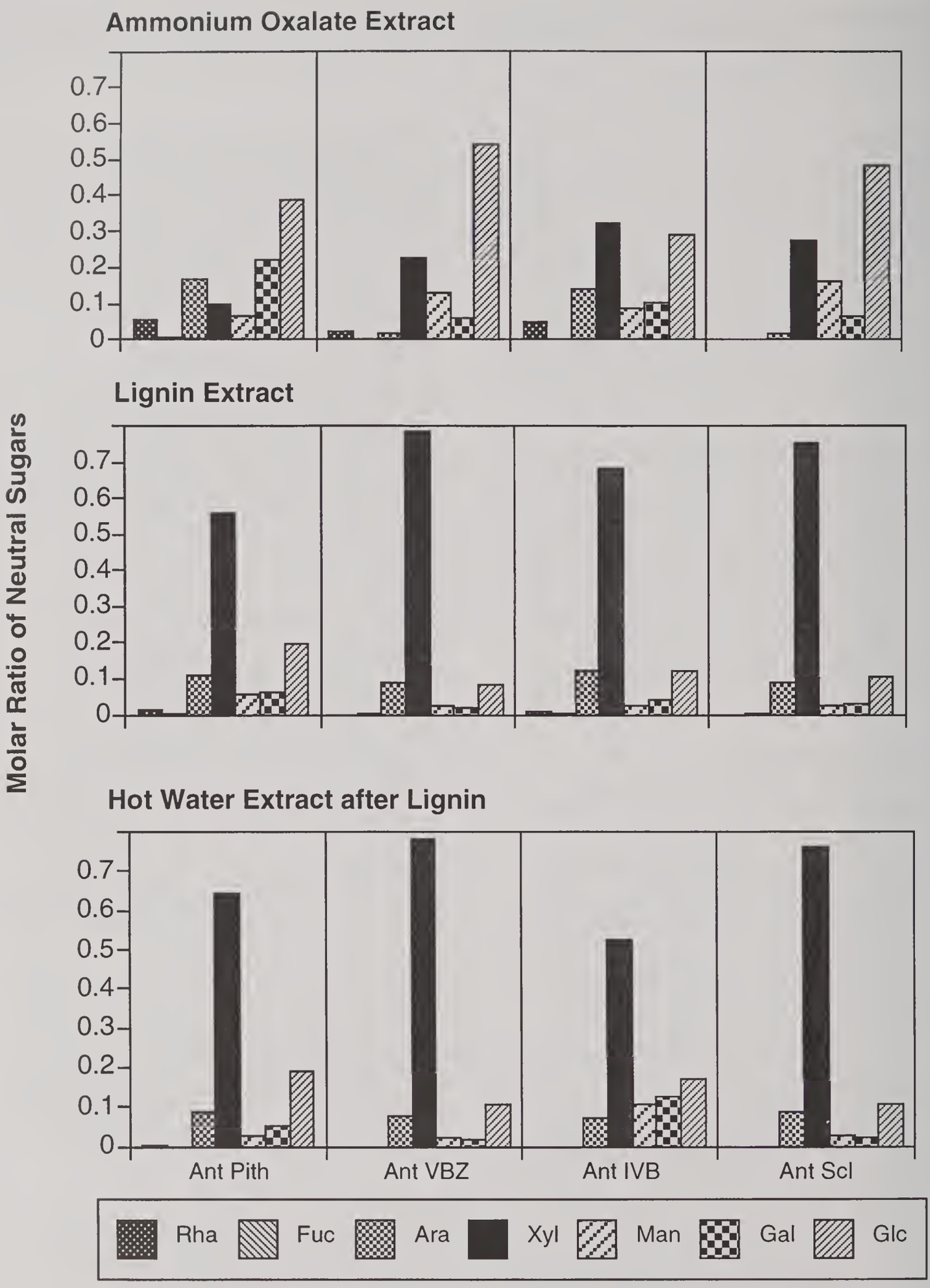


Figure 1. Neutral sugar composition of structural polysaccharides isolated by ammonium oxalate, sodium chlorite delignification, and hot water extraction after delignification.

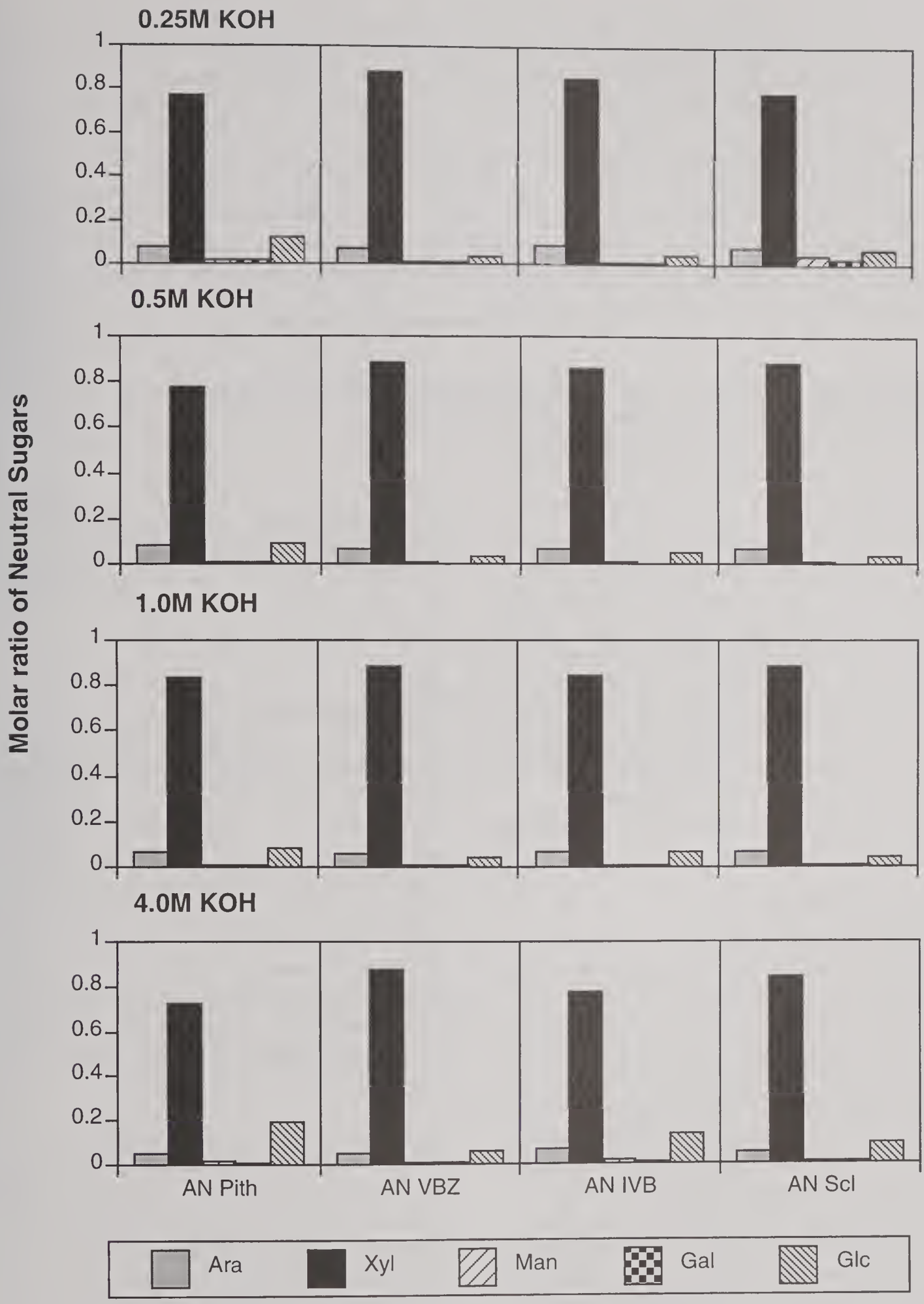


Figure 2. Neutral sugar composition of structural polysaccharides isolated by progressively increasing concentrations of KOH.

Table 1. Recovery of total carbohydrate in each of the major polysaccharide groups from the wall fractionation procedure. AN = anthesis; Pith = pith cells; VBZ = vascular bundle zone cells; IVB = inner vascular bundles, SCL = sclerenchyma.

	g/g CW			
Cell Type	Pectic ext.	KOH ext.	Cellulose	Lignin
AN Pith	0.030	0.330	0.396	0.128
AN VBZ	0.031	0.284	0.459	0.181
AN IVB	0.039	0.354	0.416	0.161
AN SCL	0.040	0.292	0.423	0.132

Determining the Role of Corn Wall Peroxidases in the Formation of Dehydrodiferulates That Cross-Link Arabinoxylans

R.D. Hatfield, B. Jones, J. Grabber and J. Ralph

Introduction

Grasses contain a significant amount of ferulic acid within their cell walls. Recently we have shown that grass walls contain most of the dehydrodimers of ferulic acid predicted from free radical coupling mechanisms (Ralph et al. 1994). The most predominate diferulates were coupled 8-8, 8-5, 8-O-4, with the 5-5 dimer being relatively minor in all samples. We are interested in knowing more about cross-linking mechanisms within wall matrices and have undertaken a project to investigate the role of wall peroxidases in forming ferulate dehydrodimers.

Materials and Methods

Peroxidases were isolated from walls or media of 14-18 day old cultures (*Zea mays,* cv. Black Mexican). Initial work revealed that the peroxidases secreted into the culture medium were the same as those extracted from isolated walls of corn cell cultures using 200mM $CaCl_2$. The crude culture media was fractionated into major subgroups of peroxidases based on their isoelectric points (pI) using ammonium sulfate fractionation, DEAE anion exchange chromatography, and chromatofocusing chromatography. Fractions containing high peroxidase activity were pooled and concentrated for subsequent chromatography steps. Specific activity against methyl ferulate, methyl *p*-coumarate, methyl sinapate, sinapyl alcohol, and coniferyl alchol was determined spectrophotometrically (see Table 1 for conditions). Product formation was determined using methyl ferulate as the peroxidase substrate and analyzing for dehydrodimers.

Results

The crude peroxidase mixture was separated into several subfractions using combinations of anion exchange and chromatofocusing chromatography. This fractionation scheme did not produce single protein bands but rather 2-4 closely related peroxidases in each subfraction. Four subfractions were selected for further study–P1, P2, P3 and PNa. The isoelectric points of the subgroups based on IEF PAGE 3-10 gels using diaminobenzidine to stain for peroxidase activity were: P1 pH 8-9 (2 protein bands), P2 pH 6.5-8 (4 protein bands), P3 pH 6 (1 protein band) and PNa pH 3-4 (4 protein bands). The pH optimum for each of the subgroups was nearly the same–5 to 5.5.

Activity of the peroxidases using different substrates varied with the substitution upon the aromatic ring (Table 1). For all peroxidase groups, the highest activity was against methyl ferulate, followed by coniferyl alcohol. Both of these have a methoxyl group at C3 on the aromatic ring. Methyl *p*-coumarate (no methoxyl substitution) was also actively utilized by the peroxidases but at roughly

half the rate of the methyl ferulate and coniferyl alcohol (Table 1). Methyl sinapate and sinapyl alcohol (methoxyl substitution at both C3 and C5 of the aromatic ring) were poorly utilized by all of the peroxidases tested (Table 1).

Methyl ferulate was used as a substrate to determine if the peroxidase subgroups produced different dehydrodimers. For each subgroup of peroxidases, the dehydrodiferulates detected arose from radical coupling reactions involving C8 and producing dehydrodiferulates coupled at 8-8, 8-O-4 and 8-5. No detectable 5-5 or 4-*O*-5 dimers were produced by the peroxidases. Although 5-5 dimers have been identified in plant extracts, no 4-*O*-5 dehydrodiferulate has been detected.

Conclusions

All peroxidase groups readily utilized the methyl ferulate to produce the same dehydrodimers. For methyl ferulate, 8-coupled products are the preferred radical coupling mechanism for forming dehydrodimers. Within the wall matrix, positioning of xylan chains may aid in 5-5 dehydrodiferulate formation. Alternatively, the 5-5 product may readily undergo additional radical coupling to form trimers in the free solution state, therefore being lost to detection. All of the dimers could potentially undergo additional radical reactions forming higher order products accounting for the decrease in total recovery of methyl ferulate (unreacted + dimers) with increased reaction time.

Reference

Ralph, J., S. Quideau, J.H. Grabber and R.D. Hatfield. 1994. Identification and synthesis of new ferulic dehydrodimers present in grass cell walls. J. Chem. Soc., Perkin Trans. 1. 23:3485-3498.

Table 1. Substrates utilized by the peroxidase subgroups. All values are the amount of substrate used in mmoles/min/mg of protein. (Reaction contained 0.15 mM substrate, 0.25 mM H_2O_2, in 1 mL of 50 mM Tris -acetate pH 5)

Peroxidase subgroup	Methyl ferulate	Methyl *p*-coumarate	Methyl sinapate	Coniferyl alcohol	Sinapyl alcohol
Pna	564	328	2	564	12
P1	148	51	0.2	116	2.7
P2	75	21	0.1	54	0.1
P3	14	11	0	10	0.6

Table 2. Reaction products produced from peroxidase subgroups utilizing methyl ferulate as substrate (0.15mM Me-FA, 0.25 mM H_2O_2 in 5 mL of 50 mM Tris-acetate pH 5). Values are the fractional amounts produced of each of the dehydrodimers. No 5-5 or 4-*O*-5 dimers were detected.

	Dehydrodimer linkage		
Peroxidase subgroup	8-5	8-*O*-4	8-8
Pna	0.36	0.27	0.38
P1	0.25	0.38	0.37
P2	0.29	0.36	0.36
P3	0.31	0.08	0.61

Syringaldazine Oxidase and Phenylalanine Ammonia Lyase Activities in Relation to Lignin Deposition in Legumes

J.E. Bidlack, D.R. Buxton, R.M. Shibles and I.C. Anderson

Introduction

Increased knowledge of the temporal relationship between enzyme activity and cell-wall deposition will improve our understanding of wall biosynthesis and structure and help provide a foundation for targeted manipulation to improve wall digestibility. Among cell-wall components, lignin deserves attention because it provides plants with mechanical strength, protection against pests, and is thought to provide resistance to digestion by ruminant animals. Lignin deposition in the wall occurs during its thickening after the cell has completed elongation. Phenylalanine ammonia lyase (PAL), a general phenylpropanoid enzyme, and syringaldazine oxidase (SAO), a specific peroxidase, catalyze the first and last steps of the lignin biosynthetic pathway, respectively. The objectives of this investigation were to: 1) determine the temporal relationships of lignin deposition and PAL activity in relation to SAO activity, and 2) determine if SAO activity is more closely related to lignin content than PAL activity.

Materials and Methods

'Arrow' alfalfa, 'Viking' birdsfoot trefoil, and 'Arlington' red clover were grown in a greenhouse in 3.8 L pots with four replicates. The basal 10 cm of forage stems were sampled at 14, 28, 42, 56, and 70 days during regrowth. Acetone powders were prepared and analyzed for PAL and SAO activities. Cell-wall components, including lignin, were also determined. Enzyme activity was calculated on both a protein and a plant basis. Concentrations of cell-wall components over time were fitted with the Gompertz function and enzyme activities were fitted with a third-order quadratic equation. Times of maximum deposition were determined by setting the second derivative of the Gompertz function equal to 0 and solving for t (time). Times of maximum PAL and SAO activities were determined by setting the first derivative of the quadratic equal to 0 and solving for t.

Results and Discussion

Activities of PAL initially increased followed by decreased or plateaued activity as a function of regrowth days (Fig. 1). Significant decreases after peak activity of PAL were more apparent than the slight decreases of SAO activity after 25 days of growth. Neither PAL nor SAO activity on a protein basis were correlated with lignin content; however, PAL on a per plant basis was correlated with lignin content across species and SAO on a per plant basis was correlated with lignin content within species. Alfalfa, which consistently had the highest lignin content throughout the regrowth period, also exhibited higher wall-component depositions compared with the other species. Activity of PAL was also higher in alfalfa, but SAO activity of

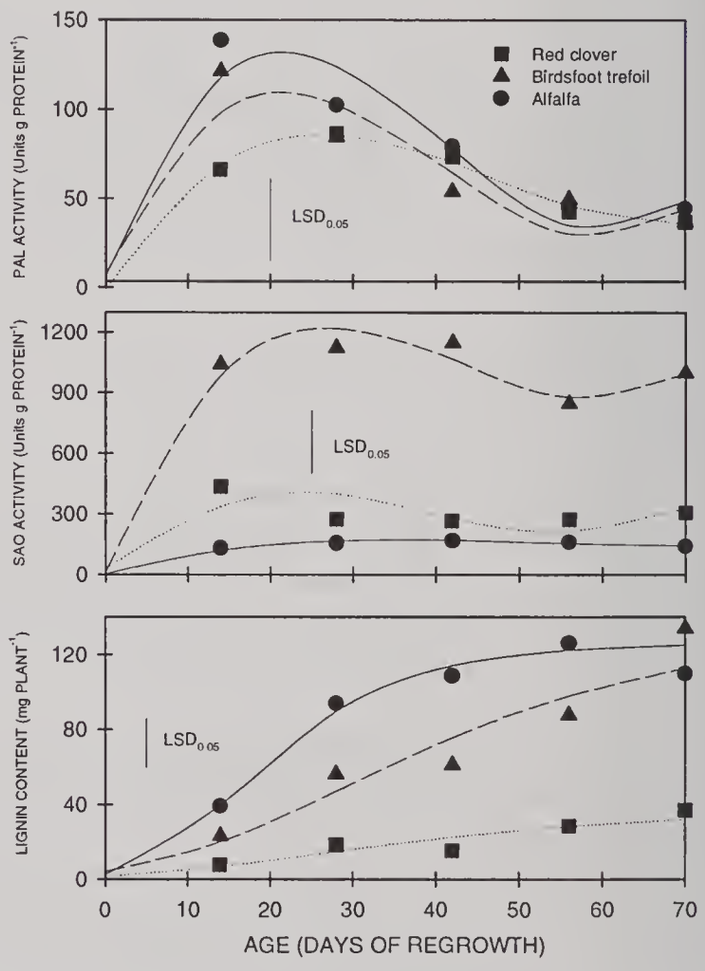


Figure 1. PAL and SAO activities and lignin content of basal stems of three legumes

birdsfoot trefoil was at least three-fold higher than that of alfalfa throughout the growth period.

Time and extent of maximum lignin deposition in red clover lagged behind maximum deposition in alfalfa and birdsfoot trefoil (Table 1). Among cell-wall components, maximum hemicellulose deposition occurred first, followed by cellulose (1 to 3 days later) and then lignin (up to 14 days after maximum hemicellulose deposition). Maximum PAL activity preceded maximum SAO activity by 2 to 12 days.

Table 1. Time of maximum cell-wall component deposition on a per-plant basis and maximum PAL and SAO activities on a per protein basis in stems of three forage legumes.

	Species		
Measurement	Alfalfa	Birdsfoot trefoil	Red clover
	- - - - - - - - - days - - - - - - - - -		
Hemicellulose	13.0	25.5	20.6
Cellulose	14.7	28.7	23.9
PAL activity	20.3	21.5	25.3
SAO activity	32.1	26.4	27.3
Lignin	17.4	30.0	34.0

Conclusions

Both PAL and SAO are related to lignin deposition on a per plant basis with maximum activity occurring first in PAL and then in SAO several days later.

Cell-Wall Composition of Corn Internodes of Varying Maturity

T.A. Morrison, H.G. Jung, D.R. Buxton and R.D. Hatfield

Introduction

Lignification of the cell wall during normal plant development is likely the primary limitation to microbial fermentation of wall polysaccharides in the rumen of cattle. A simple reduction in lignin concentration may be a limited option for improving digestibility because lignin provides the structural scaffold for the plant and has a defensive role in avoiding pathogen invasion and environmental stress. Our research seeks to identify key steps in lignification that can maximize wall polysaccharide digestibility while avoiding reduction in agronomic fitness of plants. The molecular composition of the lignin polymer may account for much of the wall's resistance to microbial attack while in the rumen. We examined the relationship of cell-wall constituents to stage of tissue and internode development of corn. This information provides a better understanding of the sequential deposition of wall components and changes in cross-linkage patterns of polysaccharides to lignin during wall development.

Materials and Methods

Rind and pith internode tissues were separated from corn plants grown in a growth chamber and harvested at the 15th leaf stage of development. Determinations were made of cell-wall neutral sugars, uronic acids, Klason lignin, hydroxycinnamic acids, and the syringyl-to-guaiacyl ratio (S/G) of lignin.

Results and Discussion

From the youngest internode (I13) to the oldest (I7), cell-wall concentration increased continually in the rind tissue, but in the pith tissue, concentrations increased through I10, and then plateaued (Fig. 1). Commensurate with these changes, the lignin proportion of the wall increased from about 75 to 140 g kg^{-1} and neutral sugar and uronic acid proportions of the wall decreased from 836 and 101 g kg^{-1} to 759 and 36 g kg^{-1}, respectively. Internode rind vascular tissue, which lignified earlier and to a greater extent than pith material, had significantly higher levels of Klason

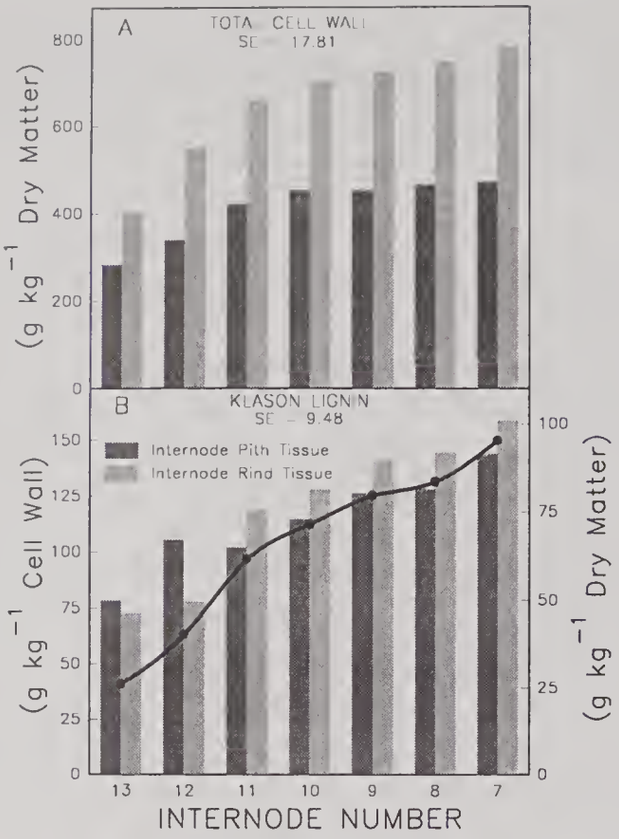


Figure 1. Concentrations of total cell wall (a) and Klason lignin (b) in the pith and rind of Internodes 13 through 7 of corn. Spline plots represent concentration of Klason lignin on a g kg^{-1} dry matter basis.

lignin and ferulic acid (FA) and *p*-coumaric acid (PCA) esters. There was a marked increase in PCA ester concentration with internode maturity (Fig. 2) and this pattern mirrored a steady rise in the S/G ratio of the lignin. Ferulate ester levels were high in young internodes, but concentrations declined in more mature internodes (Fig. 3). The ether-linked form of FA increased sharply during the early stages of internode lignification, but then also declined.

Conclusions

Composition of lignin changes during cell development with lignin deposited later in lignification being richer in syringyl units. Neutral sugar and uronic acid proportions of total wall fall with internode maturation. The change in FA linkage form with cellular development is consistent with the hypothesis that FA esters of arabinoxylan are laid down early and incorporated into the developing and elongating primary wall. Ferulic acid then becomes linked to the growing lignin polymer, thereby cross-linking lignin to polysaccharide during secondary wall thickening.

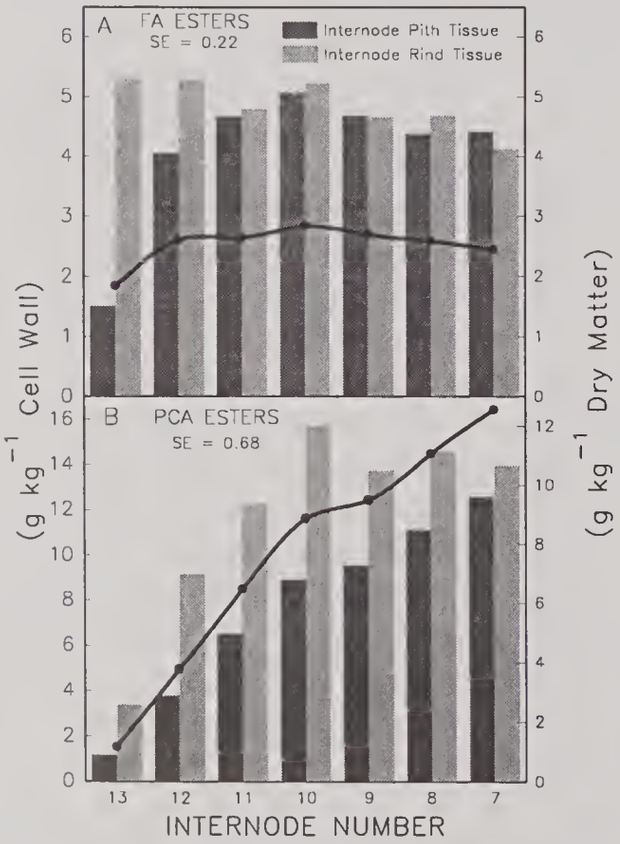


Figure 2. Concentrations of FA (a) and PCA (b) in the pith and rind of Internodes 13 through 7 of corn. Spline plots represent concentrations on a g kg^{-1} dry matter basis.

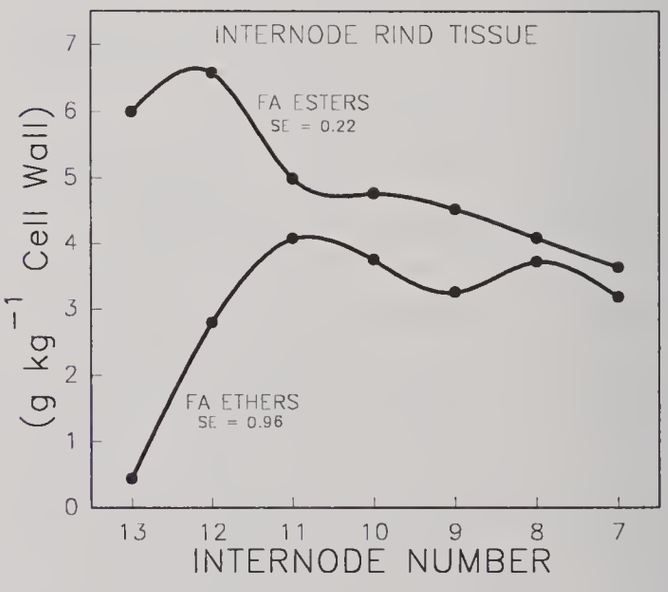


Figure 3. Concentrations of FA esters and ethers in the rind tissues of Internodes 13 through 7 of corn.

Competition Among Different Species of Ruminal Cellulolytic Bacteria in Cellobiose-Limited Chemostats

Y. Shi and P.J. Weimer

Introduction

Cellulose is the major component of forage cell walls, and its digestion by ruminal microorganisms provides the bulk of the volatile fatty acids (VFA) used by the forage-fed ruminant animal for energy and milk production. Ruminal cellulose digestion is thought to be mediated primarily by three bacterial species: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus*. The most commonly studied laboratory strains of these three species display quantitatively similar cellulolytic capabilities. However, because these species produce different ratios of fermentation endproducts, the relative populations and/or activities of these species may have profound effects on VFA ratios in the rumen. Little is known about the interactions among these species, particularly under conditions of substrate limitation that predominate in the rumen. The purpose of this study was to directly determine the ability of three predominant ruminal cellulolytic bacteria to compete with one another for growth-limiting concentrations of cellobiose, an important intermediate in the enzymatic depolymerization of cellulose and cellodextrins.

Methods

F. succinogenes S85, *R. flavefaciens* FD-1, and *R. albus* 7 were used for growth experiments. Continuous cultures were performed at 39°C under CO_2 in a stirred reactor (working volume 139 mL) continuously fed a modified Dehority medium supplemented with cellobiose (4 g/L) as sole energy source and with yeast extract (1 g/L). Two types of experiments were conducted: co-inoculation experiments in which two species were mixed aseptically and then inoculated into the reactor and "challenge" experiments in which one species was added to a steady-state chemostat culture of another species. For all experiments, samples were withdrawn from the reactor at various times for measurement of residual soluble sugars (by the anthrone colorimetric reaction), soluble fermentation products (by HPLC), and cell mass (estimated from nitrogen content of cell pellets). Populations of individual species were determined in three ways: i) by characteristic fermentation products (succinate for S85 or FD-1, ethanol for 7); ii) by signature membrane fatty acids (pentadecanoic acid for S85, 13-methyltetradecanoic acid for FD-1, and hexadecanoic acid for 7); and iii) using oligonucleotide probes homologous to characteristic sequences of 16S rRNA. RNA isolated from each sample was slot blotted on nylon membranes, hybridized to digoxigenin-labeled probe, and reacted with chemiluminescent substrate (Boehringer-Mannheim Genius system). X-ray film exposed by the chemiluminescent reaction was scanned by a densitometer.

Results

The outcomes of the different coculture experiments are summarized in Table 1. As expected from its higher affinity (lower $S_{0.5\mu max}$ value) for cellobiose, *R. flavefaciens* FD-1 readily outcompeted *F. succinogenes* S85, regardless of whether both species were co-inoculated into the chemostat or FD-1 was added to an established steady-state culture of S85.

R. albus 7 outcompeted *F. succinogenes* S85 (Fig.1) or *R. flavefaciens* FD-1, despite its poorer affinity for cellobiose. The success of 7 against S85 is not due to production of an inhibitor, as both 7 and S85 grew to approximately equal populations when co-inoculated into batch cultures. Instead, it appears that 7 was able to adapt to more rapid growth at lower cellobiose concentrations. The takeover of a steady-state culture of S85 by 7 was

accompanied by a reduction in the steady-state concentrations of soluble sugars from 0.63 mM glucose equivalents in the S85 monoculture to 0.49 mM glucose equivalents at steady state in the 7-dominated culture. Moreover, the selected population recovered from the chemostat at the end of the run had a μ_{max} similar to the original 7 culture (0.48 h^{-1}), but its $S_{0.5\mu max}$ value (0.23 mM) was five-fold lower than that of the original 7 culture (1.21 mM).

The mechanism underlying the success of 7 against FD-1 remains to be elucidated. Another *R. albus* strain (8) is known to produce an inhibitor of FD-1, and the success of 7 against FD-1 may be due to production of an inhibitor as well. However, co-inoculation of 7 and FD-1 into vials containing an excess of cellobiose has produced inconsistent results (monocultures of 7, or binary cultures in which both species are present in substantial quantities).

Conclusions

R. flavefaciens FD-1 outcompetes *F. succinogenes* S85 for cellobiose by a classical pure and simple competition mechanism based on its higher affinity for this substrate. *R. albus* 7 outcompetes S85, apparently due to selection in the chemostat of a population of 7 with a higher affinity for cellobiose. *R. albus* 7 also outcompetes *R. flavefaciens* FD-1, but it is not yet clear if this interaction involves the production of an inhibitor.

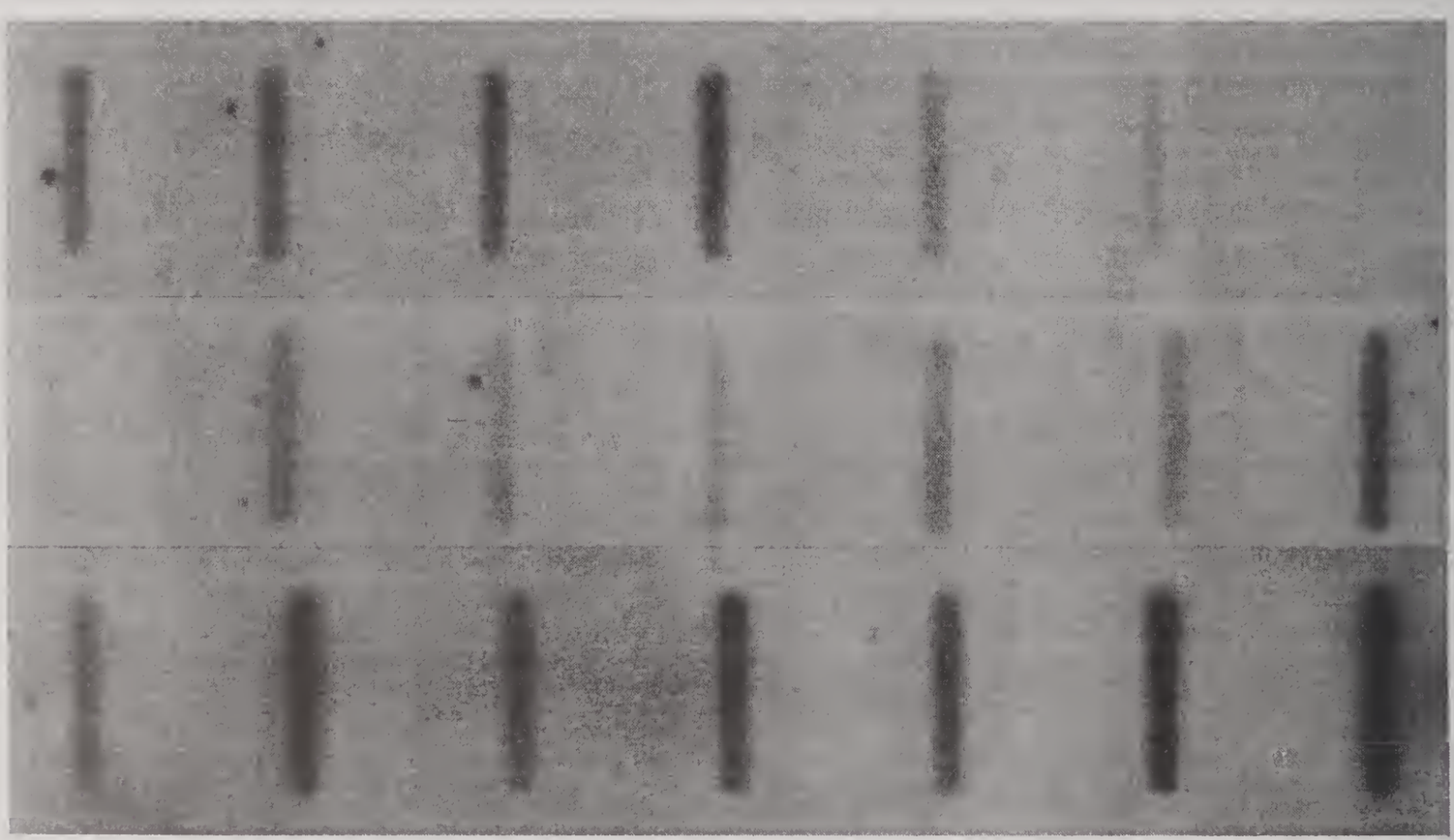


Figure 1. Cell populations in a cellobiose-limited chemostat culture (D = 0.07 h^{-1}) of F. succinogenes S85 challenged with R. albus 7 at 47 hours. Top panel: Slot blot of 5 ng of RNA from culture samples at indicated times, detected by chemiluminescence after hybridization with oligonucleotide probes. S85 A3c = F. succinogenes probe; RAL196 = R. albus probe; EUB338 = universal eubacterial probe that detects both species. Bottom panel: Estimate of cell populations in the chemostat, determined from RNA-specific oligonucleotide probes (RNA) or from membrane fatty acid composition of cell pellets (MFA).

Table 1. Outcome of binary cocultures of *F. succinogenes* S85, *R. flavefaciens* FD-1, and *R. albus* 7 in cellobiose-limited chemostats.[a]

Inoculation order	D (h^{-1})[b]	pH	Cellobiose (mM glucose equiv.) Initial	1st st.st.[c]	2nd st.st.[d]	Dominant strain	Assay method and lower detection limit[e]
Co-inoculation:							
S85 + FD-1	0.067	6.24	17.85	2.28	—	FD-1	MFA (4.5)
7 + FD-1	0.024	6.36	24.51	0.24	—	7	FP (0.4), MFA (4.5), RNA (1.0)
7 + S85	0.070	6.40	23.12	0.76	—	7	FP (0.2), MFA (10.9), RNA (1.0)
Sequential:							
S85, then FD-1	0.088	6.73	6.09	0.39	0.13	FD-1	MFA (3.1), RNA (1.0)
FD-1, then S85	0.169	6.62	16.98	1.09	1.09	FD-1	MFA (3.1), RNA (1.0)
FD-1, then 7	0.067	6.49	14.26	0.96	1.36	7	FP (0.8), MFA (3.2), RNA (1.0)
7, then FD-1	0.167	6.71	12.89	0.09	0.09	7	FP (0.8), MFA (3.2), RNA (1.0)
S85, then 7	0.070	6.36	23.12	0.63	0.49	7	FP (0.2), MFA (10.9), RNA (1.0)
7, then S85	0.166	6.54	12.89	0.08	0.08	7	FP (0.4), MFA (10.9), RNA (1.0)

[a]Steady-state data only. Complete time-course data not shown.
[b]Dilution rate in reciprocal hours.
[c]Steady-state before challenge with second strain
[d]Steady-state after challenge with second strain
[e]Assay method: MFA = signature membrane fatty acids; FP = characteristic fermentation products; RNA = oligonucleotide probes to species-specific 16S rRNA. Values in parentheses indicate minimum percentage of the population of the subordinate strain detectable by the method.

Competition Among Different Species of Ruminal Cellulolytic Bacteria in Cellulose-Limited Chemostats

Y. Shi and P.J. Weimer

Introduction

Cellulose is the major component of forage cell walls, and its digestion by ruminal microorganisms provides the bulk of the volatile fatty acids (VFA) used by the forage-fed ruminant animal for energy and milk production. Ruminal cellulose digestion is thought to be mediated primarily by three bacterial species: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus*. The most commonly studied laboratory strains of these three species display quantitatively similar cellulolytic capabilities. However, because these species produce different ratios of fermentation endproducts, the relative populations and/or activities of these species may have profound effects on VFA ratios in the rumen. Little is known about the interactions among these species, particularly under conditions of substrate limitation that predominate in the rumen. The purpose of this study was to examine directly the competition among these three cellulolytic species for growth rate-limiting concentrations of cellulose.

Methods

F. succinogenes S85, *R. flavefaciens* FD-1, and *R. albus* 7 were used for growth experiments. Continuous cultures were performed at 39°C under CO_2 in a stirred reactor (working volume 875 mL) continuously fed a modified Dehority medium supplemented with cellulose (4.4 - 7.1 g/L) as sole energy source. The cellulose-containing medium was delivered as a CO_2-segmented slurry to prevent settling of cellulose in the pump lines. Two types of experiments were conducted: co-inoculation experiments in which two species were mixed aseptically and then inoculated into the reactor and "challenge" experiments in which one species was added to a steady-state chemostat culture of another species. Samples were withdrawn at various times and were analyzed for residual cellulose by a modified NDF method, and for soluble sugars, fermentation endproducts, and total cell mass as described in the previous report. The relative amount of each bacterial species was determined by three different methods (characteristic fermentation products, characteristic membrane fatty acids, and oligonucleotide probes homologous to 16S rRNA sequences specific for each species) as described in the previous report.

Results

The outcomes of the different coculture experiments are summarized in Table 1. *R. flavefaciens* FD-1 supplanted *F. succinogenes* S85 regardless of whether the strains were co-inoculated or an established steady-state culture of S85 was challenged with FD-1. The results are consistent with our previous observations that FD-1 adheres more rapidly and completely to cellulose and has a higher affinity for all cellodextrins (G2-G6) tested. Although S85 is capable of growth on glucose, the free glucose concentration in the culture was insufficient to sustain growth at the dilution rate of the chemostat.

Under the conditions tested, *R. albus* 7 was able to co-exist in binary culture with either *F. succinogenes* S85 or *R. flavefaciens* FD-1. In the case of FD-1, this co-existence may reflect a balance between factors favoring FD-1 (more rapid adherence to cellulose and more favorable growth kinetics on low concentrations of cellodextrins) and those favoring 7 (possible production of an inhibitor). In the case of S85, the coexistence with 7 is more complex. These two strains, in many cases, have similar growth rates on equivalent concentrations of cellodextrins and similar in vitro kinetics of adherence to cellulose, although growing cultures of S85 always show more complete attachment of cells to cellulose than do growing cultures of 7. *R. albus* 7 may benefit from the active efflux of longer chain cellodextrins that is routinely performed by S85.

Conclusions

Competition studies in cellulose-limited chemostats reveal that *R. flavefaciens* FD-1 readily outcompetes *F. succinogenes* for limiting amounts

of cellulose, probably due to its more avid adherence to cellulose and its more rapid growth on the products of cellulose hydrolysis. By contrast, *R. albus* 7 was able to co-exist with either of the other two species, suggesting that the interactions among these species are not based on pure and simple competition for cellulose, but instead reflect a combination of physiological characteristics of these species.

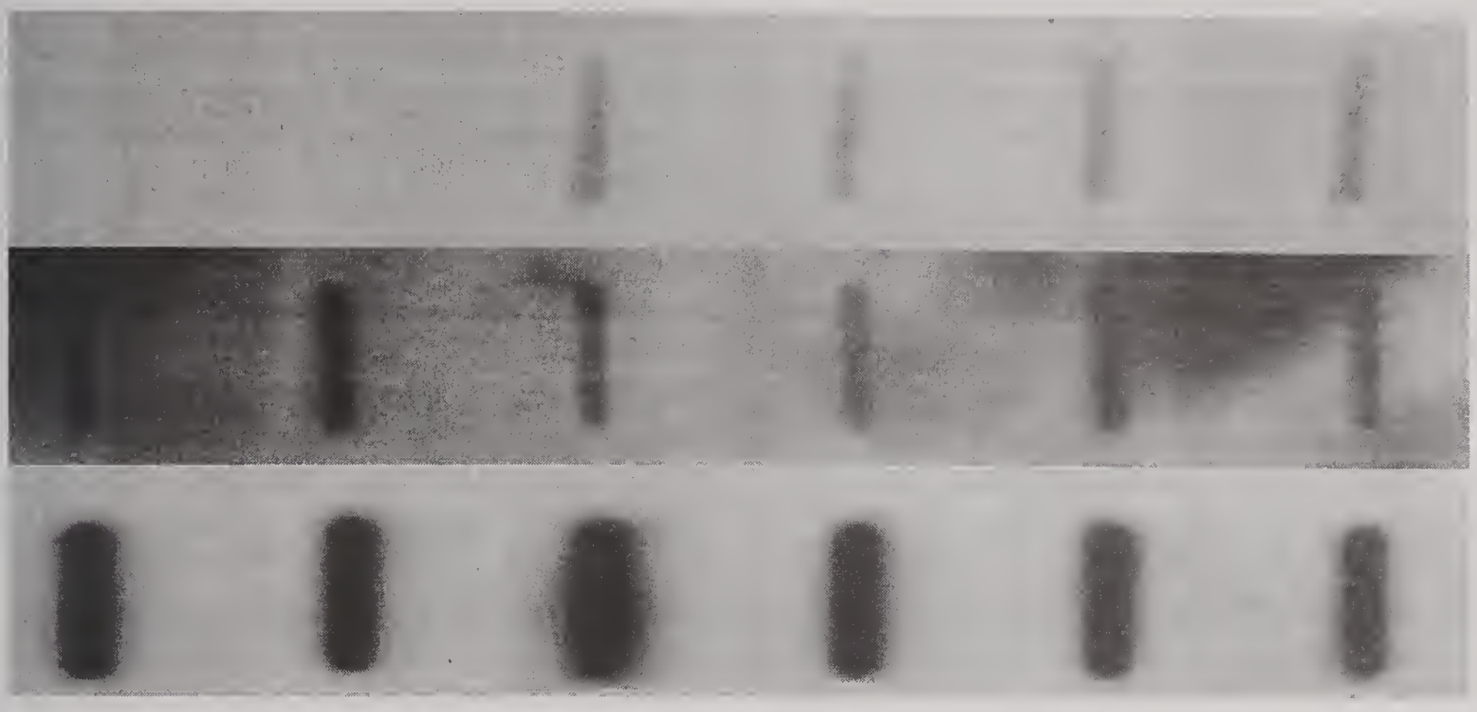


Figure 1. Cell populations in a cellulose-limited chemostat culture (D = 0.03 h^{-1}) of F. succinogenes S85 challenged with R. albus 7 at 90 hours. Top panel: Slot blot of 5 ng of RNA from culture samples at indicated times, detected by chemiluminescence after hybridization with oligonucleotide probes. A3c = F. succinogenes probe; RAL196 = R. albus probe; EUB338 = universal eubacterial probe that detects both species. Bottom panel: Estimate of cell populations in the chemostat, determined from RNA-specific oligonucleotide probes (RNA) or from membrane fatty acid composition of cell pellets (MFA).

Table 1. Outcome of binary cocultures of *F. succinogenes* S85, *R. flavefaciens* FD-1, and *R. albus* 7 in cellulose-limited chemostats.[a]

Inoculation order	D (h^{-1})[b]	pH	Residual cellulose (g/L) Initial	Steady-state	Culture composition[c] S85	FD-1	7	Assay method and lower detection limit[d]
Co-inoculation:								
S85 + FD-1	0.049	6.44	4.50	1.23	< 4.5	> 95.5	—	MFA (4.5)
7 + FD-1	0.016	6.79	7.08	6.55	—	85.1	14.9	RNA (1.0)
7 + S85	0.024	6.27	4.44	2.39	89.9	—	10.1	RNA (1.0)
7 + S85	0.020	6.10	5.59	3.53	78.1	—	21.9	RNA (1.0)
Sequential:								
S85, then FD-1	0.030	6.48	4.35	1.42	< 4.5	> 95.5	—	MFA (4.5)
FD-1, then 7	0.029	6.52	5.22	1.78	—	89.2	10.7	RNA (1.0)
					—	94.3	5.7	MFA (3.2)
S85, then 7	0.031	6.50	5.66	1.40	90.7	—	9.3	RNA (1.0)

[a]Steady-state data only. Complete time-course data not shown.
[b]Dilution rate in reciprocal hours.
[c]Estimated composition at end of incubation, calculated from characteristic biomarkers indicated in (d).
[d]Assay method: MFA = signature membrane fatty acids; RNA = oligonucleotide probes to species-specific 16S rRNA. Values in parentheses indicate minimum percentage of the population of the subordinate strain detectable by the method.

Predicted Outcome of Competition Among Ruminal Cellulolytic Bacteria for Soluble Products of Cellulose Digestion

Y. Shi and P.J. Weimer

Introduction

Cellulose is the major component of forages and its digestion and subsequent fermentation by ruminal microbes provides much of the energy for forage-fed ruminants. Ruminal degradation of cellulose is mediated primarily by cell-associated enzymes produced by a few predominant bacterial species. The primary products of cellulose depolymerization are the soluble β-linked glucosyl oligomers (cellodextrins) cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and cellohexaose (G6). Of these, only cellobiose is commercially available in quantities needed for growth studies. Previously, we described the isolation of these cellodextrins in quantities sufficient for growth studies and determined the maximum growth rates (μ_{max}) and substrate concentrations permitting half-maximal growth ($S_{0.5\mu max}$)for two predominant cellulolytic species, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*. This report summarizes data for the third major cellulolytic species, *R. albus*, and uses these growth parameters to evaluate the probable outcome of competition for each substrate by the three cellulolytic species.

Methods

Mixed cellodextrins were prepared by hydrolysis of Sigmacell 20 microcrystalline cellulose with fuming HCl. Individual cellodextrins (G2-G6) were separated by charcoal/Celite/stearic acid column chromatography using a stepwise ethanol gradient. Identities of individual cellodextrins were determined by analytical HPLC using a Bio-Rad 42A Carbohydrate column eluted with H_2O. Growth experiments were conducted with glucose (G1) and each individual cellodextrin at 39°C in Balch-type anaerobic tubes containing a modified Dehority medium supplemented with various concentrations of each cellodextrin. Growth was monitored turbidimetrically at 600 nm, and growth rates determined as the linear portion of the regression line of ln (A_{600}) versus time. Maximum growth rates and substrate concentrations permitting growth at half-maximal rate were determined from Monod plots (1/μ vs. 1/S). Two to four separate experiments were conducted for each cellodextrin/culture combination, with paired tubes for each cellodextrin concentration within each experiment.

Results

R. albus 7 appears to cleave cellodextrins of three or more glucosyl units prior to uptake, as has been noted for *R. flavefaciens* FD-1 and *F. succinogenes* S85. The Monod kinetic parameters for *R. albus* 7 (Table 1) indicate that this strain has μ_{max} values on most cellodextrins somewhat similar to those of the other two cellulolytic strains. While the values of $S_{0.5\mu max}$ are somewhat higher than for the other two strains for G1 and G2, there is a dramatic reduction in these values with increasing chain length.

By fitting the values of μ_{max} and $S_{0.5\mu max}$ to the Monod equation

$$\mu = \mu_{max} (S / S + S_{0.5\mu max}),$$

the growth rate of all three cellulolytic species could be predicted for each soluble sugar substrate (Fig. 1). The data indicate that *R. flavefaciens* FD-1 displays an ability to grow on each cellodextrin more rapidly than the other two species and that *F. succinogenes* S85 can generally grow more rapidly on glucose (a substrate not utilized by FD-1) and on most cellodextrins than can *R. albus* 7.

Table 1. Monod kinetic parameters for *R. albus* 7 grown on glucose and cellodextrins

Substrate	μ_{max} (h^{-1})	$S_{0.5\mu max}$ (mM)
G1	0.58[a]	4.16[a]
G2	0.48[a]	1.21[b]
G3	0.61[a]	0.41[c]
G4	0.53[a]	0.54[c]
G5	0.46[a]	0.20[d]

[a,b,c,d]Values within the same column having different superscripts differ significantly *(P < .05)*.

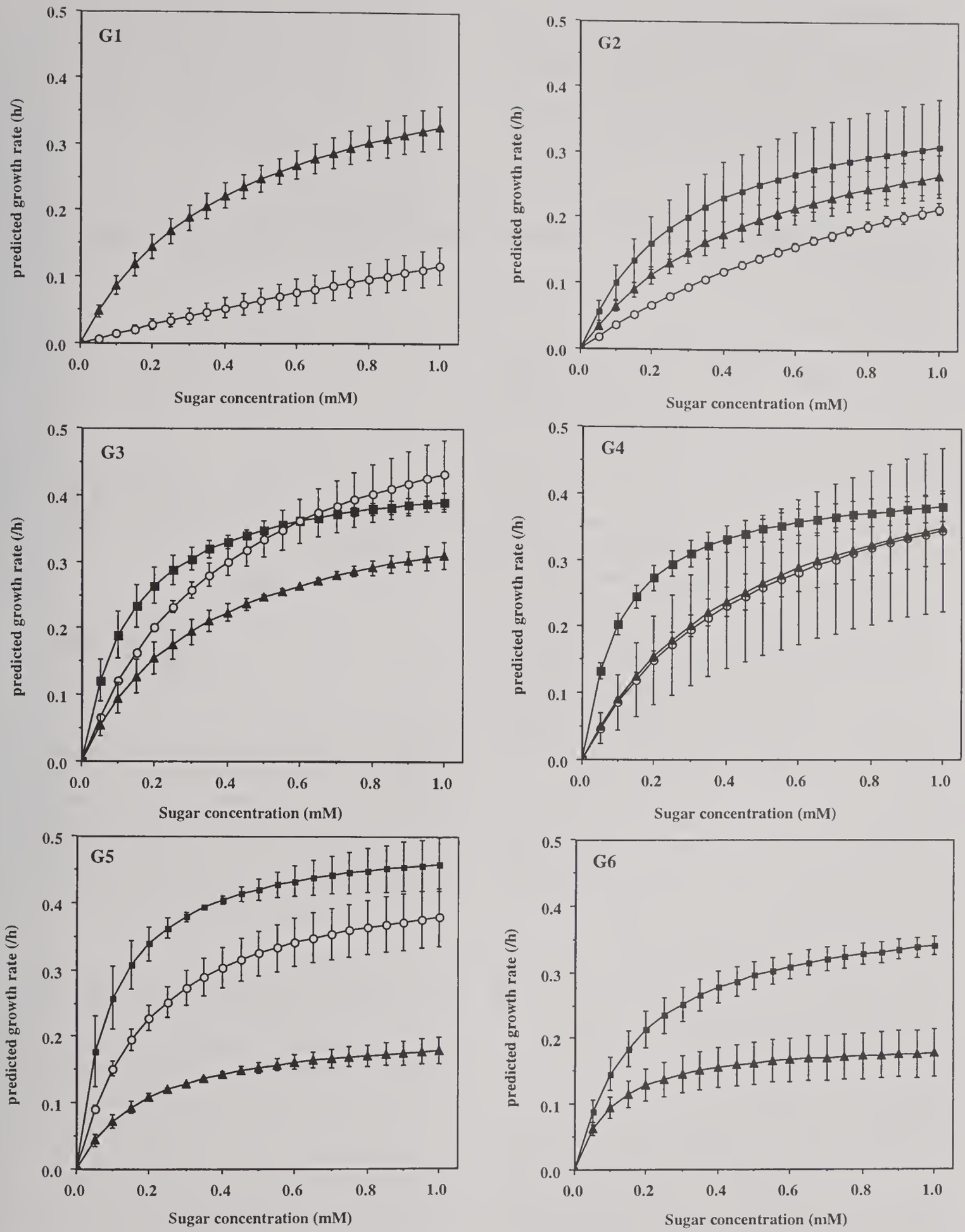


Figure 1. Predicted growth rates of three predominant ruminal cellulolytic bacteria, calculated from the Monod equation and the experimentally-determined values of μ_{max} and $S_{0.5\mu max}$. Error bars indicate 90% confidence intervals. ■, R. flavefaciens FD-1; ○, R. albus 7, ▲, F. succinogenes S85

Conclusions

The data suggest that *R. flavefaciens* FD-1 should outcompete *F. succinogenes* S85 and *R. albus* 7 for all cellodextrins regardless of their concentration if their interactions were based on pure and simple competition for soluble cellodextrins. While *F. succinogenes* should theoretically outcompete *R. albus* 7 for glucose, the low growth rate and poor affinity for this substrate relative to other glucose utilizing ruminal bacteria make glucose utilization by ruminal cellulolytic bacteria in the rumen environment unlikely.

Direct Measurement of the Penetration of Ruminal Bacteria into the Lumina of Maize Sclerenchyma

P.J. Weimer

Introduction

Matrix interactions among cell wall polymers are generally believed to be the major factor limiting forage digestion by ruminal microorganisms. Research from several laboratories over the last fifteen years has suggested that the architecture of certain plant cell types may also be an important determinant of digestibility. Electron microscopic evidence suggests that plant cell walls are digested from the interior (luminal) face, apparently because of the recalcitrance of the highly lignified middle lamellae that separate the outer face of the cell walls from one another. Consequently, the ability of fibrolytic microbes to reach the interior of the plant cell — a process thought to occur primarily by diffusion — may be particularly important in long, narrow cell types such as sclerenchyma, whose cells may have lengths up to 1 mm, but luminal diameters of only a few µm. Theoretical calculations of Wilson and Mertens (1995) suggest that a bacterium of 1 mm diameter will require 4.3 days to penetrate halfway down a sclerenchyma cell of 0.5 mm length. However, direct measurements of this penetration have not been made, owing to the lack of a suitable method. The purpose of this study was to develop a suitable method for measurement of this penetration.

Theory and Methods

In principle, the penetration of cells into a porous or capillary-like structure may be determined by a solute exclusion method. This technique (Fig. 1) was originally developed by Stone and Scallan in 1968 to characterize the pore structure of wood pulps. The method uses a series of macromolecular probes (each having its own diameter) as "feeler gauges" to determine the pore diameter and total pore volume of wetted biomass material. Probes having diameters smaller than the diameters of the pores will enter the pores by diffusion, resulting in the displacement of pore water and consequent dilution of the probe solution, which can be measured by optical methods (light scattering or optical rotation). Materials that have proven successful as probes include dextrans and polyethylene glycols. For this study the method was modifed to use cells of the ruminal cellulolytic bacterium *Fibrobacter succinogenes* S85 as the probe.

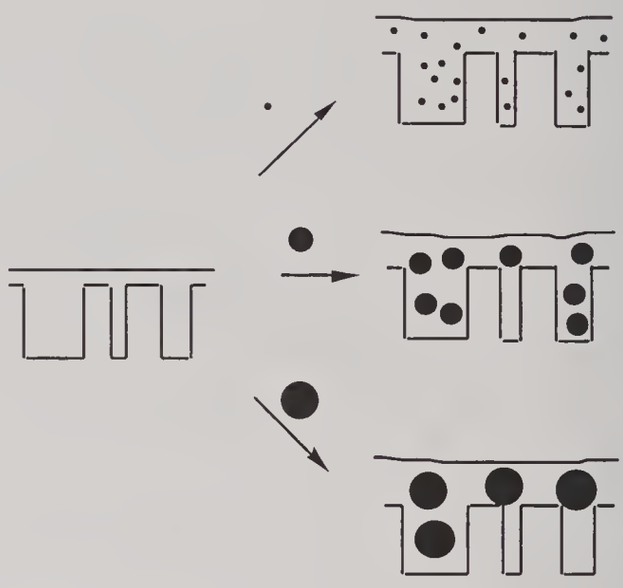


Figure 1. Schematic of the solute exclusion (Stone and Scallan 1968) technique for measuring the pore structure of wood pulps. The displacement of water resulting from diffusive entry of a molecular probe causes measurable decrease in probe concentration in the bulk liquid phase. The measurements are performed with multiple subsamples using a variety of different probes of known molecular size. Application of the data to a series of equations permits estimation of the sizes of the entire population of pores.

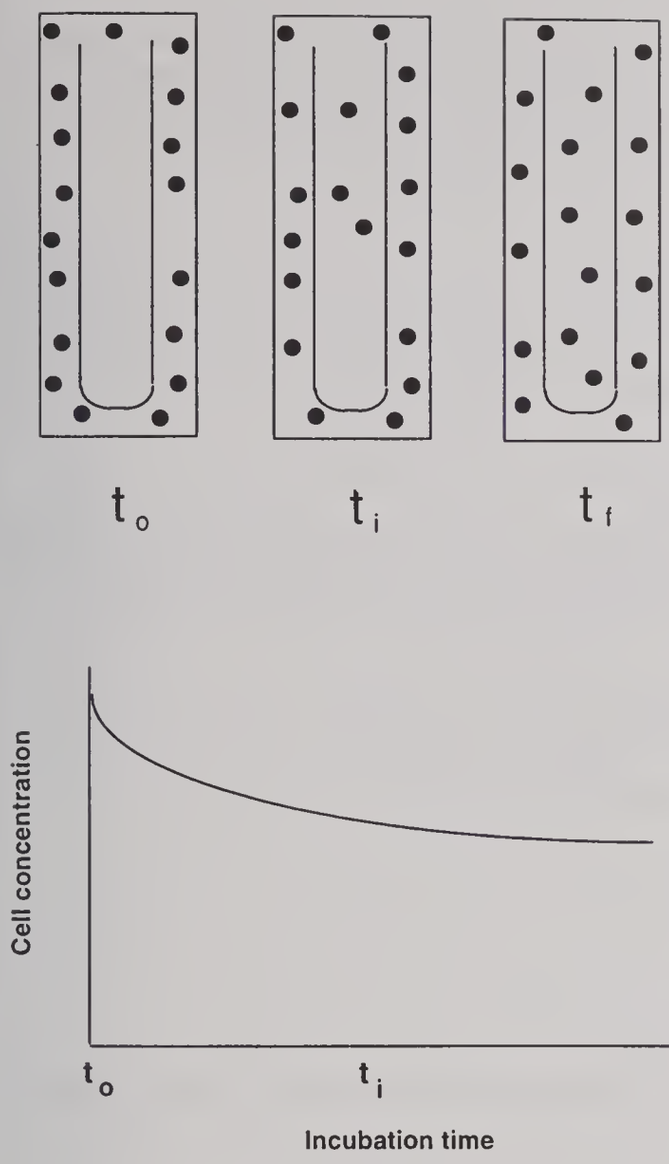


Figure 2. Modification of the solute exclusion technique to measure the rate of entry of bacterial cells into the lumina of isolated forage cell types. Top panel: Bacterial cells (illustrated as dark spheres) are allowed to displace water in the lumina of sclerenchyma cells (indicated by the open tube-like structures, not drawn to linear scale). Bottom panel: The kinetics of dilution of the original cell suspension can be related to the rate of entry of the bacterial cells.

The original solute exclusion technique is an equilibrium method in which the diffusion of probes into the pores continues until the concentration of the probe in the inaccessible water reaches a constant value (i.e., when influx into the pores equals efflux out of the pores). However, in this study the method was modified to measure the rate of probe dilution with time. This was possible because the bacterial cells (~ 1 µm diameter) are much larger than macromolecular probes and thus will diffuse into the lumina only (not pores within the plant cell wall), and at a rate that will be much slower and thus useful for kinetic studies. One of the required characteristics of the probes is that they do not physically or chemically interact with the biomass substrate. This characteristic would appear to exclude the use of cells of the ruminal cellulolytic bacteria as probes because they avidly bind to cellulose. Fortunately, this difficulty can be overcome by conducting the experiments in the presence of methylcellulose, a soluble cellulose ether that prevents binding of cells to the cellulosic substrate. Addition of formaldehyde to the culture as a fixative prevents both an artifactual increase in cell density that would result from growth, and artifactual decrease in cell density that would result from lysis of starving cells.

Dried maize sclerenchyma (25 mg, isolated by the method of Grabber and Jung) was equilibrated in MCF solution (0.1% methylcellulose / 1% formaldehyde (v/v) in water) for 48 h, after which the solution was withdrawn with a Pasteur pipette. The wetted sclerenchyma was then resuspended in 3.00 mL of MCF containing freshly harvested cells of the ruminal cellulolytic bacterium *Fibrobacter succinogenes* S85(A_{525} ~ 0.35). Vials were incubated with gentle (60 rpm) orbital shaking. At various intervals, 1.00 mL samples of solution

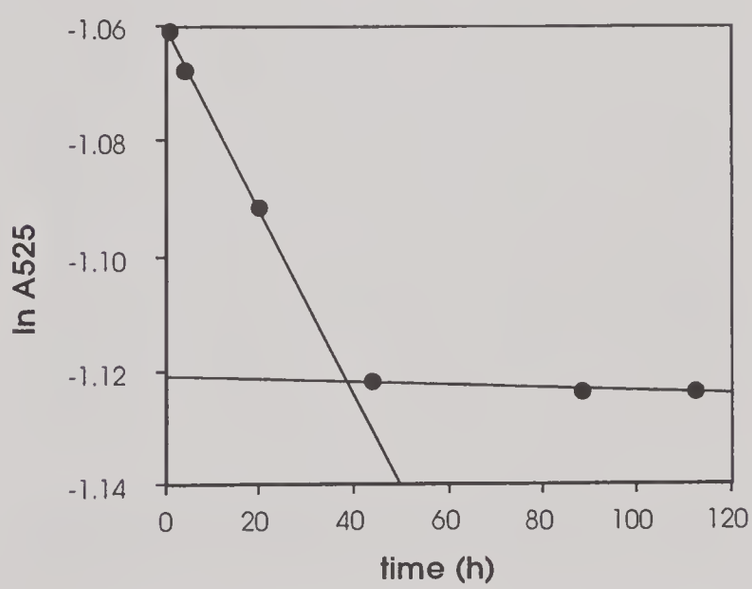


Figure 3. Diffusion of F. succinogenes S85 cells into maize sclerenchyma tissue. The time course is plotted as two lines corresponding to an initial first-order rate of dilution (r^2 = 0.999) and a plateau region where dilution reaches equilibrium (r^2 = 0.768).

were withdrawn from the vials, taking care not to disturb the rapidly settling sclerenchyma, and the absorbance of the solutions measured at 525 nm. Samples were returned to the vials after each absorbance reading.

Results

Typical results are shown in Fig. 3. Equilibration of cell density (the indicator of penetration of cells into the sclerenchyma) reaches equilibrium by about 40 h. This penetration, while somewhat faster than that predicted by Mertens and Wilson (1995), nevertheless represents over half of the retention time of large particles in the rumen. Thus, it appears that penetration of nonmotile fibrolytic bacteria into the lumina of sclerenchyma is slow and is a limitation to digestion in the rumen.

Conclusion

Direct measurement of the rate of diffusion of bacterial cells into sclerenchyma tissue is possible via modification of the solute exclusion technique. These measurements indicate that the diffusion process is a potential limiting factor in the digestion of sclerenchyma tissue.

Reference

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The Effect of Amino Nitrogen on the Energetics of Ruminal Bacteria and Its Impact on Energy Spilling

J.S. Van Kessel and J.B. Russell

Introduction

Ruminant feeding systems have generally been based on the assumption that energy should be given first consideration, but there is increasing evidence that amino acid supply can in many cases limit the production of high producing ruminants. Recent work has stressed the importance of 'escape' or 'bypass' protein in meeting amino acid requirements, but microbial protein is generally the dominant source of amino acids passing into the small intestine. The 1989 NRC recommendations for dairy cattle used a static equation to predict microbial protein flow from the rumen and assumed that ruminal microorganisms always had the same growth efficiency.

Most bacteria can utilize ammonia as a nitrogen source, but many bacteria prefer preformed amino acids. The impact of preformed amino acids on the growth efficiency of ruminal bacteria has not been clear-cut. In vitro batch cultures of mixed ruminal bacteria grew 60 to 400% more efficiently when amino acids or peptides, respectively, were provided, but the responses of carbohydrate-limited cultures were much less or even negligible. Some workers noted a 30% increase in microbial flow from the rumen when casein was added to a urea-based diet, but ruminally degraded protein has, in many cases, provided no benefit.

Materials and Methods

Ruminal contents were squeezed through cheesecloth. After gas production had buoyed small feed particles to the top and protozoa had settled to the bottom, bacteria were anaerobically transferred from the center of the flask to a Hungate tube. The ruminal fluid was diluted 10^8-fold in basal media containing salts, cysteine, sulfide vitamins, minerals, volatile fatty acids, protein hydrolysate, soluble starch, cellobiose, sucrose, xylose,

arabinose, and pectin. These predominant ruminal bacteria were grown in batch and continuous culture. Optical density was measured at 600 nm (18 mm tubes). Carbohydrate disappearance from cell-free supernatants was measured by the anthrone method. Fermentation acids were determined by high pressure liquid chromatography. Cell protein was measured by the folin method. Cellular polysaccharide was determined by the anthrone method.

Results and Discussion

Predominant ruminal bacteria (PRB) that were obtained from a 10^8 dilution of ruminal fluid could be maintained as a mixed population for long periods of time, so long as they were provided with a complex mixture of carbohydrates. Carbohydrate-limited, ammonia-excess continuous cultures (.07/h) of PRB had a low maintenance energy requirement, but the non-growth energy dissipation of ammonia-limited, carbohydrate-excess PRB was approximately 10-fold higher (.96 versus .09 mg of hexose equivalent per mg of protein per h, respectively). Mathematical derivations indicated that this additional non-growth energy dissipation could be accommodated by a growth-rate independent energy spilling function. Peptides and amino acids had little impact on the yield of carbohydrate-limited, ammonia-excess continuous cultures (.07 /h), but amino N caused a large increase in the growth rate and yield of energy-excess batch cultures. The amino N-dependent change in growth rate and yield indicated that the energy-excess batch cultures had the same capacity to spill energy as ammonia-limited, carbohydrate-excess PRB (.80 versus .86 mg of hexose equivalent per mg of protein per h, respectively). When the energy-excess batch cultures were provided with amino N, the growth rate increased, the difference between anabolic and catabolic rates was smaller, and less energy was spilled.

In the derivation of Pirt, the yield (Y) of a bacterium can be predicted from the maintenance energy coefficient (m), the theoretical maximum growth yield of the bacterium (Y_G), and the growth rate (μ):

$$1/Y = m/\mu + 1/Y_G.$$

Maintenance energy coefficients are typically measured under carbohydrate-limiting conditions, but carbohydrate-sufficient cultures often have a much inflated m. The variation in m was a lively subject of debate in the 1970's , and the terms "overflow metabolism," "slip reactions," "uncoupling," and "energy spilling" were all used as hypothetical explanations of the unexplained energy dissipation. Based on the observation that the apparent m of ammonia-limited cultures was higher, it was possible to modify the basic yield equation,

$$1/Y = m/\mu + 1/Y_G,$$

to include an energy spilling function (e):

$$1/Y = m/\mu + e/\mu + 1/Y_G,$$

where e is defined as the slope of the carbohydrate-excess Pirt plot minus the slope of energy-limited cells (m).

The Cornell Net Carbohydrate and Protein System (CNCPS) uses m and Y_G to predict the Y of ruminal bacteria . All ruminal bacteria are assigned the same Y_G, but the m of nonstructural carbohydrate-fermenting (NSC) bacteria is 3-fold greater than the m of structural carbohydrate-fermenting (SC) bacteria (.3 and .1 mg of carbohydrate per mg of protein per h, respectively). In this regard, the yield of NSC bacteria is discounted by a higher m, but these bacteria are stimulated by amino N availability. The CNCPS predicts μ from the first order rate of carbohydrate degradation (Kd),

$$\mu = Kd,$$

but the effect of N on μ is ignored. If N is limiting:

$$\mu < Kd.$$

The CNCPS adjusts the Y of NSC bacteria with an empirical peptide/amino acid stimulation function,

but this function stimulates Y even at very low Kd. Because amino N only increased the Y of PRB when carbohydrate was in excess and the μ permitted by ammonia was less than Kd, the CNCPS may be over predicting the benefit of amino N when the rate of carbohydrate fermentation is low.

Conclusions

The modification of the CNCPS to include an energy spilling term may provide a basis for estimating the microbial growth even if total N is restricted.

A Ribosomal RNA Approach for Assessing the Role of Obligate Amino Acid-Fermenting Bacteria in Ruminal Amino Acid Deamination

D.O. Krause and J.B. Russell

Introduction

Amino acid deamination in the rumen is a nutritionally wasteful process that often produces more ammonia than the bacteria can utilize. Excess ammonia is absorbed by the animal and converted to urinary urea. Ruminant nitrogen excretion is a major source of environmental pollution, and as much as 50% of the feed nitrogen can be excreted. Because ammonia can be oxidized by nitrifying bacteria, nitrite and nitrate accumulate in ground water.

Most strains of ruminal bacteria produce little ammonia. Based on the activities and numbers in the rumen, *Prevotella* (*Bacteroides*) *ruminicola* seems to be the most important ammonia-producing bacterium. Later work, however, indicates that this species cannot account for all the ammonia accumulation in vivo.

Ruminal bacteria have been traditionally isolated in media containing carbohydrates and either ammonia, or low concentrations of protein hydrolyzate, as a nitrogen source. In the late 1980's, enrichments on a high concentration of protein hydrolyzate yielded three ruminal bacteria with very high specific activities on ammonia production, and these bacteria could not utilize carbohydrates as an energy source. 16S rRNA sequence analyses indicated that these obligate amino acid-fermenting, monensin-sensitive bacteria were *Peptostreptococcus anaerobius*, *Clostridium sticklandii* and a new species designated as *Clostridium aminophilum*.

Most probable number estimates indicated that these monensin-sensitive, obligate amino acid-fermenting bacteria were less than 10% of the total ruminal count. Because the feed additive, monensin, decreased ammonia accumulation in the rumen by 50%, it appeared that obligate amino acid-fermenting bacteria were deaminating a large fraction of the ruminally degradable protein. These estimates were confounded by the fact that many ruminal bacteria cannot be cultured in the laboratory and the observation that obligate amino acid-fermenting bacteria are not the only monensin-sensitive bacteria in the rumen. Because 16S rRNA can differentiate bacteria without in vitro culturing, we used 16S rRNA probes to assess the contribution of obligate amino acid-fermenting bacteria in vitro and in vivo.

Materials and Methods

Two cows were fed chopped timothy hay (9% crude protein, 41% acid detergent fiber, 65% neutral detergent fiber, and 17% nonstructural carbohydrates) with a rotary feeder every two hours. Monensin was added to the diet to achieve a daily dose of 350 mg/day. Ruminal bacteria were grown anaerobically at 39°C in a basal medium containing salts, cysteine, sulfide, vitamins and minerals, 2-mercaptoethanesulfonic acid, volatile fatty acids, Trypticase and carbohydrates.

Aligned 16S rRNA nucleic acid sequences were obtained from the Ribosomal Data Base and probe sequences that had at least 3 to 4 mismatches in comparison to their closest phylogenetic relatives

were chosen. rRNA was extracted by bead-beating and denatured by glutaraldehyde. The rRNA sample was diluted with polyadenylic acid and bromophenol blue, and applied to nylon membranes in a slot-blot apparatus. Membranes were air dried and then baked at 120°C for 30 min. Samples were prehybridized at room temperature for 2 h. Digoxygenen labeled probes were added to the hybridization solution and allowed to incubate at 22°C for 16 h. The relative abundance of 16S rRNA was determined with a universal probe.

Ammonia was analyzed by the indophenol method. Cell protein was measured by the folin method. Fermentation acids and carbohydrates were determined by high-performance liquid chromatography.

Results and Discussion

The 16S rRNA probes indicated that monensin inhibited the growth of *P. anaerobius* and *C. sticklandii* in the rumen. *C. aminophilum* was monensin-sensitive in vitro, but *C. aminophilum* persisted in the rumen after monensin was added to the diet. An in vitro culture system was developed to assess the competition of *C. aminophilum, P. anaerobius* and *C. sticklandii* with predominant ruminal bacteria (PRB). PRB were isolated from a 10^8 dilution of ruminal fluid and maintained as a mixed population with a mixture of carbohydrates. PRB did not hybridize with the probes to *C. aminophilum*, *P. anaerobius* or *C. sticklandii*. PRB deaminated Trypticase in continuous culture, but the addition of *C. aminophilum*, *P. anaerobius* and *C. sticklandii* caused a more than 2-fold increase in the steady state concentration of ammonia. *C. aminophilum*, *P. anaerobius* and *C. sticklandii* accounted for less than 5% of the total 16S rRNA and microbial protein. Monensin eliminated *P. anaerobius* and *C. sticklandii* from the continuous cultures, but it could not inhibit *C. aminophilum*. The monensin-resistance of *C. aminophilum* was a growth-rate dependent, inoculum size-independent phenomenon that could not be maintained in batch culture. Based on these results, the feed additive, monensin, cannot entirely counteract the wasteful amino acid deamination of obligate amino acid-fermenting ruminal bacteria.

The monensin-resistance of *C. aminophilum* reduces the amino acid sparing effect of monensin. Based on a bacterial percentage of 1.4, a bacterial protein concentration of 1.1 mg/ml, a yield for *C. aminophilum* of 3.3 mg protein/mmol amino acid fermented, a molecular weight of 100 for an average amino acid, a fluid dilution rate of 0.07 h^{-1}, a ruminal volume of 70,000 ml, the additional loss of amino acids due to *C. aminophilum* would be 55 g of amino acids/day. Since the total protein intakes of the cows were 630 g/day, it appeared that *C. aminophilum* might be wasting approximately 9% of the feed protein.

For more than 25 years, ruminant nutritionists tried to "manipulate" ruminal fermentation with chemical additives, but the successes have often been accidental. Truly mechanistic studies of ruminal ecology are confounded by the complexity of ruminal bacteria, the labor intensity of traditional enumeration techniques, and the inability of rumen microbiologists to cultivate all of the ruminal bacteria. Many of these problems can be circumvented by 16S rRNA analyses, but even these techniques do not negate the high cost of animal experimentation, animal variation and the virtually infinite combination of dietary ingredients.

Conclusions

In vitro systems do not mimic all aspects of ruminal fermentation but if properly designed can offer a mechanistic foundation for subsequent animal trials.

The Cellular Location of the *Prevotella ruminicola* β 1,4-D-Endoglucanase and Its Occurrence in Other Strains of Ruminal Bacteria

R.G. Gardner, J.E. Wells, J.B. Russell and D.B. Wilson

Introduction

Ruminant animals have developed the capacity to digest cellulose by exploiting a symbiotic relationship with cellulolytic ruminal bacteria. Enumeration studies have indicated that non-cellulolytic bacteria outnumbered the cellulolytics even when wheat straw was the only ingredient in the diet, and later work indicated that there was a crossfeeding of "cellulose fragments" from cellulolytic to non-cellulolytic bacteria. *Prevotella ruminicola* is usually described as a starch-degrading bacterium, but it is able to utilize water soluble cellodextrins, and some strains have considerable carboxymethylcellulase (CMCase) activity.

The CMCase of *Prevotella ruminicola* B_14 degrades CMC at a rapid rate, but it lacks a cellulose-binding domain and cannot degrade crystalline, acid-swollen or ball-milled cellulose at a significant rate. The addition of a cellulose-binding domain to the C-terminus of the CMCase increased the rate of native cellulose digestion 10-fold, and this finding has been used as a basis for creating a cellulolytic bacterium that can digest cellulose at low pH.

Materials and Methods

P. ruminicola strains TC1-1, TF1-3 and TS1-5 were provided by H. J. Flint. *P. ruminicola* strains 23, 118B, 20-78, 20-63, M384, GA-33, 2202 and D42f were provided by M. Cotta. *B. ovatus*, *B. distasonis*, *R. amylophilus*, and *S. ruminantium* were provided by T. Miller. The bacteria were grown anaerobically in a semi-defined medium.

Cells were harvested by centrifugation. The cell suspension was sonicated, and cell debris was removed by centrifugation. The whole cells, cell-free supernatants and cell-extracts were assayed for CMCase, mannanase and xylanase activities. Samples were incubated at 55°C for 20 min and loaded onto 8% SDS polyacrylamide gels containing 2% polysaccharide. Western immunoblotting was performed using antisera prepared against the 40.5kDa cloned CMCase purified from *E. coli*. Antiserum was reacted with whole cells to insure that it did not contain antibodies against other *P. ruminicola* proteins.

The CMCase was purified via a scheme employing ammonium sulfate, phenyl sepharose, Hypatite C and Q-sepharose. The CMCase-containing fractions were concentrated using a 30,000 MW cutoff filter.

Results and Discussion

Prevotella ruminicola B_14, TC1-1, TF1-3 and TS1-5 all produced immunologically crossreactive 88 kDa and 82 kDa CMCases. *P. rumincola* 23, 118B, 20-63 and 20-78 had much lower CMCase activities and Western blots showed no cross-reaction with the B_14 CMCase antiserum. *Fibrobacter succinogenes* S85 and *Selenomonas ruminantium* HD4 and D produced CMCases, but these enzymes were smaller and did not cross-react with the B_14 CMCase antiserum. The B_14 CMCase antiserum inhibited the B_14, TC1-1, TF1-3 and TS1-5 CMCase activities and agglutinated these cells, but it had no effect on the other strains or species. Based on these results, the B_14 CMCase is a strain specific enzyme that is located on the outside surface of the cells. *P. ruminicola* B_14 cultures grown on sucrose did not have significant CMCase activity, but these cells could bind purified 88 and 82 kDa CMCase, but not 40.5 kDa CMCase. Because the 40.5 kDa CMCase is a fully active, truncated form of the CMCase, it appears that the N-terminal domain of the 88 kDa B_14 CMCase anchors the CMCase to the cells. Cells grown on cellobiose produced at least 10-fold more CMCase than the sucrose-grown cells, and the cellobiose-grown cells could only bind 15% as much CMCase as sucrose-grown cells. Virtually all of the CMCase activity of exponentially growing cultures was cell-associated, but CMCase activity

was eventually detected in the culture supernatant. Based on the observation that the 88 kDa CMCase was gradually converted to the 82 kDa CMCase when cultures reached stationary phase without a change in specific activity, it appears that the 82 kDa protein is probably a proteolytic degradation product of the 88 kDa CMCase.

The CMCase hydrolyzed carboxymethylcellulose and barley glucan but not xylan or mannan. The activity varied 20-fold when *P. ruminicola* $B_1$4 was grown on different sugars. The highest activities were observed with mannose, cellobiose or xylose and little activity was observed with sucrose, arabinose or rhamnose. Because cells that were grown with sucrose in addition to mannose or sucrose in addition to cellobiose had at least 10-fold less activity than cells grown on mannose or cellobiose, respectively, it appeared that sucrose acts as a repressor. Arabinose also decreased activity but not to as great an extent as sucrose. The complexity of endoglucanase expression was also illustrated by the observation that the combination of two inducers (mannose and cellobiose) gave at least 5-fold less activity than a single inducer (cellobiose or mannose). *P. ruminicola* $B_1$4 also had significant xylanase and mannanase activities, but these activities were present in proteins that had lower molecular weights than the endoglucanase, and these proteins did not cross react with antibody made against the endoglucanase. Mannanase activity has a similar pattern of expression to the endoglucanase, while the xylanase was not induced or repressed by the same sugars or combinations of sugars. The xylanase activity was greatest when xylan was the energy source for growth, but xylose was a very poor inducer of xylanase activity.

Conclusions

Genetic modification of the *P. ruminicola* $B_1$4 CMCase is still a reasonable mechanism for creating an acid-resistant cellulolytic ruminal bacterium.

Near Infrared Spectroscopy to Estimate Microbial Dry Matter and Nitrogen Contamination of In Situ Forage Residues

V.D. Peltekova, G.A. Broderick, A. Alexandrov, S. Atanassova and N. Todorov

Introduction

The in situ method is used widely to determine ruminal degradability of feed CP and DM. It is an attractive alternative to in vivo methods because it is simple, inexpensive, and rapid. However, several factors influence in situ results, the most important of which is microbial contamination of feed residues after incubation in the rumen. Degradation of forage CP and DM is systematically underestimated because of microbial contamination. Markers have been used to quantify microbial contamination of in situ residues. Microbial DM contamination ranged from 8 - 13% for forages and 8 - 25% for concentrates. Crude protein contamination of in situ residues was greater for forages than for concentrates. Microbial CP contamination of in situ forage residues was reported to be 15 - 50% when ^{35}S was used as the marker and 46 - 95% when diaminopimelic acid was used as the marker. Use of microbial markers to measure microbial contamination is time consuming and expensive. It may be possible to quantify microbial DM and N contamination in in situ forage residues using near infrared spectroscopy (NIRS). Our objectives were to: 1) determine the extent of microbial N and DM contamination of in situ residues of ruminally incubated ^{15}N labeled alfalfa hay, and 2) evaluate the use of NIRS to quantify microbial contamination of in situ forage residues.

Materials and Methods

Alfalfa was labeled with the stable isotope ^{15}N by growing plants on soil fertilized with $(^{15}NH_4)_2SO_4$. Plants were harvested at late bud stage, air-dried to prepare hay, and ground through a 2-mm screen.

Enrichment of ^{15}N in alfalfa hay and in situ residues was determined by isotope-ratio mass spectrometry. Average ^{15}N enrichment in the alfalfa hay used in the study was .992 ± .01 (SD) atom % excess. In situ trials were conducted in a ruminally cannulated Holstein cow fed a total mixed diet containing forage from alfalfa silage and corn silage. About 7.5 g of alfalfa hay DM was weighed into 10 cm x 20 cm dacron bags with 40 µm pore sizes and incubated in the rumen for 0, 6, 12, 24, and 48 h. All bags were soaked in tap water to remove soluble or filterable material; 0-h bags were immersed in water only. After removal from the rumen, bags were rinsed, washed, then dried. Microbial N (RMN) contamination (% of total N) was calculated from the ^{15}N enrichment in the residue, relative to the enrichment of original feed:

RMN, % = [1 - (^{15}N in residual N /^{15}N in original N)] x 100

Contaminating microbial DM was calculated from RMN assuming 7.6% N in microbial DM. The quantity of microbial DM or CP contaminating in situ residues was subtracted from total DM or CP to obtain corrected degradabilities. An iterative least squares procedure was used to fit degradability data to the equations of Ørskov and McDonald (1979). An NIR System 4250 scanning monochromator was used for the NIRS analysis on the 96 samples of dried in situ residues; reflectance spectra were recorded over the 1618 to 2320 nm region and data were transformed and analyzed. Cross validation was used to estimate validation errors; validation errors were combined into a standard error of cross validation (SECV). The factors considered in the final selection of a calibration equation included: 1) high R^2, low SECV and low bias in the validation set; and 2) high r^2 and low standard error of calibration (SEC).

Results and Discussion

Degradation of alfalfa was very rapid and most of the DM and CP disappeared during the first 6 to 12 h of ruminal incubation (Table 1). At 48 h, apparent DM and CP recoveries were 20.1 and 43.8%, respectively. Higher apparent recoveries of DM and CP, compared to recoveries corrected for microbial contamination, reflected the considerable, increasing microbial attachment and growth on the in situ residues over the incubation. The data for uncorrected residual CP could not be fit to the degradation model; however, after correcting for microbial N contamination, residual CP data fit the model well (Table 1). Apparent CP recovery was greater than corrected CP recovery ($P < .001$) and depended on the length of incubation (Table 1). Microbial N contamination of alfalfa residues increased continuously during the incubation, ranging from about 23% (6 h) to nearly 50% (48 h) of residual N (Fig. 1). The proportion of residual CP contamination was much higher than that observed for DM. This is probably due to the greater CP content of attached microbes relative to forage residues. Olubobokun and Craig (1990) reported that the CP content of firmly attached microbes was 45% of DM and undigested forage residues contained only 14.1% CP. Apparent and corrected CP degradations were 48.2 and 63.6%, respectively, ($P < .001$); apparent and corrected DM digestiblities were 70.9 and 72.7% and were not different ($P > .05$).

The range of residual microbial N, CP and DM recoveries, residual N and ^{15}N enrichment of the samples used for NIRS calibration, are in Table 2. Results from statistical analysis of NIRS calibrations and predictions of the degradation

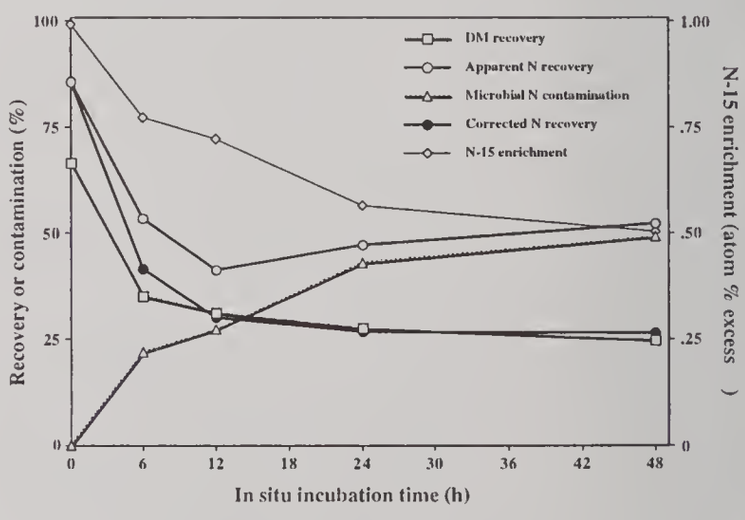

Figure 1. Apparent DM recovery, apparent and corrected N recovery, and microbial N contamination and N-15 enrichment of residues from alfalfa hay incubated in the rumen in situ.

parameters are in Table 3. The results indicated that prediction of microbial N contamination using NIRS was successful: coefficient of determination (r^2) for calibration = .957, SEC = 2.34% and SECV = 2.39%. The coefficient of determination (r^2) was greater than .5 for each of the nine wavelengths used in the calibration. The NIRS data explained a large proportion of the variation in the degradation of CP and DM in alfalfa hay. The coefficient of determination (r^2) was higher than .97 (Table 3). The SEC and SECV of NIRS estimation were of similar magnitude to the SD of replicates for chemical analysis and ranged from .22 to 4.98% for CP recovery and from .11 to 3.03% for DM recovery. Highest correlations between corrected DM recovery and spectral data were found at approximately the same wavelengths as for corrected CP recovery. There was a strong correlation between DM recovery and CP recovery (r^2 = .91); therefore, the same spectral information was used for predicting both.

Conclusions

Microbial contamination of in situ forage residues leads to systematic underestimation of effective CP and DM degradabilities. Because it can be calibrated for rapid and accurate chemical analyses, NIRS represents an attractive alternative for estimating microbial contamination. These data showed that, following calibration using ^{15}N-labelled alfalfa, NIRS was an accurate and rapid method for determining microbial contamination of in situ forage residues. These results indicate that NIRS analysis can be used to correct in situ estimates of microbial DM and CP degradation.

References

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Ørskov, E. R. and I. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. (Camb.) 92: 499-503.

Table 1. Apparent and corrected recovery of DM and CP in in situ residues of alfalfa hay and degradation parameters estimated using the model of Ørskov and McDonald (1979).

Incubation time, h	Dry matter		Crude protein	
	Apparent	Corrected[1]	Apparent	Corrected[1]
0	61.7 ± 3.2[a]	61.7 ± 3.2[a]	82.1 ± 7.4[a]	82.1 ± 7.4[a]
6	35.0 ± 4.9[b]	33.9 ± 2.1[b]	45.0 ± 6.1[b]	37.0 ± 4.1[b]
12	27.1 ± 7.7[c]	26.1 ± 2.4[c]	36.2 ± 3.6[c]	27.2 ± 2.2[c]
24	22.1 ± 3.4[d]	20.7 ± 2.8[d]	41.0 ± 4.3[d]	25.8 ± 1.3[d]
48	20.1 ± 4.2[e]	18.6 ± 3.7[e]	43.8 ± 5.4[bd]	24.5 ± 1.6[e]
Parameters[2]				
P, %	70.9	72.7	48.2	63.6
a, %	38.3	38.3	17.9	17.9
b, %	41.4	42.8	43.0	57.0
c, /h	.109	.117	NF[3]	.280
Lag, h	3.7	2.9	NF[3]	.5

[a,b,c,d,e]Mean recoveries (±SD) in the same column with different superscripts differ ($P < .05$).
[1]Differences between apparent and corrected data were significant for CP ($P < .001$) at all four incubation times, but not significant for DM ($P > .05$) at any incubation time.
[2]P = Effective degradability; a = rapidly soluble fraction; b = slowly degradable fraction; c = fractional rate at which b is degraded.
[3]Data did not fit the degradation model.

Table 2. Mean, range (minimum = MIN, maximum = MAX) and standard deviation (SD) of degradation parameters from alfalfa hay incubated in situ in the rumen and used in analysis of near infrared reflectance spectroscopy to determine microbial DM and N contamination.

Parameter	n	Mean	MIN	MAX	SD
Residual microbial N, % of total N	78	29.9	14.1	49.6	10.8
Apparent DM recovery, %	95	31.6	12.8	62.2	13.5
Corrected DM recovery, %	95	30.8	12.1	62.2	14.2
Apparent CP recovery, %	95	47.9	31.8	94.3	16.0
Corrected CP recovery, %	95	37.3	22.7	94.3	20.9
^{15}N enrichment, atom % excess	95	.75	.501	1.01	.15
Apparent residual N, % of total N	95	1.38	.89	2.65	.46
Corrected residual N, % of total N	95	1.07	.63	2.65	.59

Table 3. Results of near infrared reflectance spectroscopic determination of degradation parameters from alfalfa hay incubated in the rumen in situ.[1]

Parameter	Calibration		Validation		
	r^2	SEC	R^2	SECV	n
Residual microbial N, % of total N	.957	2.34	.951	2.39	6
Apparent DM recovery, %	.986	1.61	.984	1.70	4
Corrected DM recovery, %	.990	1.43	.988	1.54	4
Apparent CP recovery, %	.957	3.34	.950	3.55	4
Corrected CP recovery, %	.977	3.15	.973	3.40	6
^{15}N enrichment, atom % excess	.975	.023	.968	.026	7
Apparent residual N, % total N	.974	.074	.967	.084	6
Corrected residual N, % total N	.979	.086	.972	.097	6

[1] r^2 = Coefficient of determination; R^2 = coefficient of determination for cross validation; SEC = standard error of calibration; SECV = standard error of cross validation; n = number of outliers (samples with cross validation residuals > 2.5).

Comparing Forage Sources in Dairy Rations Containing Similar Neutral Detergent Fiber Concentrations

D.R. Mertens

Introduction

The NDF-Energy Intake System indicates that differences among forages will have little impact on intake and milk production when dairy rations are formulated to contain the same NDF concentration. This system implies that differences in forage quality can be compensated by changes in the forage to concentrate (F:C) ratio of the ration. Although NDF can differ in rate and extent of digestion, the NDF-Energy Intake System predicts that these effects are insignificant in relation to the ratio of fiber to nonfiber that is present in the ration. In effect, the system replaces fiber in the forage with nonfiber from concentrates to maintain a constant NDF intake when forage quality varies. It assumes that differences between fiber and nonfiber are critical and that differences within fiber or nonfibrous carbohydrates among sources are less important.

The system predicts that, if forage quality is different among forages and rations are formulated for a constant F:C ratio, rations with lower NDF will yield greater performance than those with higher NDF concentrations. Conversely, it predicts that, if F:C ratios are varied so that rations are formulated to contain the similar NDF concentrations, they will result in equivalent milk production irrespective of forage source. This study was designed to test this hypothesis.

Methods

Sixty Holstein cows, averaging 90 days in lactation and 35.1 kg milk/d, were blocked by parity and initial milk production and assigned to rations containing sorghum x sudan hybrid (SS), orchardgrass (OS), alfalfa (AS), wheat (WS), or corn (CS) silage. Cows were fed a covariate ration containing a mixture of all silages for 2 weeks followed by their assigned ration for 12 weeks. Total mixed rations contained approximately 8% roasted soybeans to insure that protein quality and quantity were not limiting production. They were formulated to have 31% aNDF and 18% crude protein (CP) using high moisture corn and soybean meal and contained between 30.3 and 31.4% aNDF and 17.7 to 19.1% CP based on analysis (Table 1). Samples of forage were taken daily and composited weekly for analysis. Dry matter (DM) was determined by oven drying at 55°C for 48 h, which approximates dry matter determined by toluene distillation, for measuring DM intake or oven drying at 105°C for expressing chemical composition. Crude protein was determined by the Kjeldahl method and NDF was determined using sodium sulfite and heat-stable amylase, and after ashing of residues (aNDF).

Results and Discussion

There were no differences in milk production ($P <$ .90) among forage sources over the entire trial (Table 2), although there was a week by source interaction (corn silage resulted in higher production initially and orchardgrass resulted in higher production later in the trial). Because cows were blocked by milk production and parity, there were differences in milk composition and intake among treatment groups during the covariate period. Thus, milk composition ($P <$.10) and average intake ($P <$ .40) did not differ among treatments (Table 2). Intake of concentrates varied from 8.0 kg/d for the corn silage ration to 12.8 kg/d for the wheat silage ration with no difference in milk production. This indicates that concentrates can be substituted for forages in rations to adjust diets for differences in forage quality

Conclusion

It appears that forages of differing qualities can result in equal performance if fed in rations that are formulated to contain similar NDF. Rather than feeding a fixed F:C ratio, it is recommended that

dairy rations be balanced for NDF concentration to adjust for differences in forage quality. Optimum production of 4% fat-corrected milk can be achieved when feeding a variety of forage sources by balancing rations to obtain an aNDF intake of 1.1 to 1.2% of body weight per day.

Table 1. Chemical composition of the different forage sources.

Component	Sorg x Sudan silage	Orchardgrass silage	Alfalfa silage	Wheat silage	Corn silage
Forages:					
Dry matter	40.2	44.8	57.9	51.7	42.1
Crude protein	12.8	15.5	17.2	10.2	8.3
aNDF[1]	54.8	48.4	45.2	54.4	41.6
Rations:					
Dry matter	55.0	57.4	64.9	64.2	57.6
Crude protein	18.5	17.7	17.7	19.0	19.1
aNDF[1]	31.0	31.1	31.4	30.3	30.5
Forage (% DM)	44.2	51.5	57.2	43.6	63.6

[1]Amylase treated, ash-free, neutral detergent fiber

Table 2. Production responses of dairy cows fed rations containing similar neutral detergent fiber (aNDF) from different forage sources.

Variable	SorgXSudan silage	Orchardgrass silage	Alfalfa silage	Wheat silage	Corn silage
Body weight (kg)	586.4	609.1	592.7	625.1	608.5
Dry matter intake (kg/d)	22.0	23.3	23.6	22.7	22.0
Milk production (kg/d)	32.4	33.7	33.6	33.5	34.6
Milk fat (%)	3.6	3.8	3.6	3.4	3.5
Milk protein (%)	3.1	3.1	3.0	3.0	3.1
Body weight change (kg/d)	.14	.09	.24	.10	.16

Defining Effective Fiber and Fiber Recommendations for Dairy Cows

D.R. Mertens

Introduction

Current NRC recommendations for dairy cattle provide only minimum recommendations for chemically measured fiber. However, physical characteristics of fiber can influence animal health, ruminal fermentation, animal metabolism, and milk fat production independently of the amount of chemically measured fiber. Using ground fibrous by-product feeds or finely chopped forages in rations that contain adequate amounts of fiber often results in reduced animal performance. This indicates that the effectiveness of fiber in maintaining animal health and productivity varies with its physical form.

Fiber recommendations for dairy rations can be improved by adjusting fiber for its effectiveness in maintaining chewing activity, ruminal pH, and milk fat percentage. Being a nutritional concept, it is clear that fiber effectiveness can be measured only by the animal. Although neutral detergent fiber (NDF) is a useful measurement of total fiber in feeds, it does not measure the physical characteristics of fiber related to effectiveness. The objective of this research was to (1) define physically effective NDF (peNDF), (2) derive coefficients for estimating peNDF, and (3) develop preliminary estimates of peNDF recommendations for dairy cows.

Materials and Methods

To develop the relationships among NDF, physical form of the NDF, and chewing activities of dairy cows consuming concentrates and fibrous by-product feeds, 39 experiments in which chewing activities were measured (214 cow-treatment combinations) were compiled from the literature. A system for classifying the physical form of feeds was designed based on the quantitative particle size information provided by various authors (Table 1). Feeds were assigned to a class based on the description of the feeds provided by the authors. If no particle size information was provided, feeds were assigned to the median class for that feed source.

Estimates of peNDF requirements for maintaining milk fat were made using a database containing 112 cow-treatment combinations from 26 experiments. A database containing 88 cow-treatment observations from 20 experiments was used to determine the peNDF requirement for maintaining ruminal pH. Experiments were restricted to those in which ruminal pH was measured a minimum of five times over an 8h period after feeding. To remove variation associated with experimental location, all regression models for determining requirements included a class variable for the institute at which the experiment was conducted.

Results and Discussion

Physical effectiveness of NDF was defined as the fraction of NDF that influences chewing activity and the biphasic nature of rumen contents (floating mat of large particles on a pool of small particles and liquid). Factors that are related to particle size and particle size reduction and their impact on optimal fermentation should be related to peNDF. Thus, peNDF is related to ruminal pH regulation via the production of salivary buffers during eating and rumination. It is also related to the formation of the mat in the rumen, which may be a critical component of the selective retention of fiber for digestion in the rumen and for the stimulation of rumination.

Long grass hay was observed to have the largest eating, ruminating, and total chewing activity per kg of NDF intake; therefore, it was used as the standard for calculating the physical effectiveness factors for all other NDF sources (Table 2). Physically effective NDF predicted total chewing activity with an R^2 of .66. Although this R^2 may seem low, the data base: (1) included 15 data sets that appeared to be outliers, (2) used some estimates of NDF and physical form classification, (3) contained observations on lactating and nonlactating cows, restricted and ad libitum feeding, and separate and total mixed rations, and (4) made comparisons across experiments; therefore, variation associated with measurement techniques, feeding systems, facilities, and animals is included in the overall R^2.

The product of observed NDF and the physical effectiveness factors in Table 2 estimates peNDF of a feed. Regression of peNDF (% of ration DM) versus milk fat percentage or ruminal pH was used to estimate recommendations. These equations indicate that 24.1 and 18.7% peNDF in ration DM would maintain milk fat concentration at 3.5 and 3.3%, respectively. Ruminal pH may be a better indication of ruminal health and optimal function than the maintenance of milk fat production because factors other than physical effectiveness of fiber influences milk fat synthesis. Solutions of the regression equations for maintaining an average pH of 6.2, 6.1 and 6.0 using ground corn as the concentrate source were 30.3, 26.7, and 22.1% peNDF in ration DM. Based on these results, the minimum recommendations for peNDF of dairy cows is between 19% (to maintain the milk fat percentage of early to mid lactating cows at 3.3%) and 22% of ration DM (to maintain an average ruminal pH of 6.0).

Conclusions

Fiber is an important characteristic of feeds that can be used to formulate optimal rations. Adjusting fiber for effectiveness provides a means of fine-tuning the formulation of dairy rations. A system is proposed that is based on NDF as the measure of

total chemical fiber in feeds. Adjustments for effectiveness of NDF in maintaining milk fat production and optimizing ruminal fermentation are based on the particle size and inherent characteristics of NDF that affect chewing activity. The system can provide practical guidelines for improving current fiber recommendations and serve as a framework for future research on meeting the fiber requirements of dairy cows.

Table 1. Physical form classification system used to describe feeds in published experiments.

Description	TLC[a]	Screen Size[b]	Grass hay	Grass silage	Corn silage	Alfalfa hay	Alfalfa silage	Concentrates
Long			Long					
Coarse chopped	4.8 to 8.0		Coarse	Long		Long		
Medium-coarse chopped	2.4 to 4.0		Medium	Medium	Long	Coarse		
Medium chopped	1.2 to 2.0		Short	Short	Medium	Medium	Long	
Medium-fine chopped	.6 to 1.0				Short	Short	Medium	
Fine chopped	.3 to .5						Short	
Ground or pelleted	.15 to .25		Ground Pelleted			Ground Pelleted		
Rolled								B, HMC[c]
Coarse ground or cracked		1.25						Cr. Corn[d]
Medium ground		.90						C, Complex[e]
Fine ground or pelleted		.63						Pelleted

[a]Theoretical length of cut in cm.
[b]Grinder screen aperture in cm.
[c]Barley and high moisture corn (both shelled and ear corn).
[d]Cracked corn.
[e]Ground corn and complex mixtures of fibrous protein supplements and nonforage fiber sources.

Table 2. Physical effectiveness factors for NDF in feeds of each physical form classification for eating, ruminating, and total chewing time relative to long grass hay.

Description	TLC[a]	Screen Size[b]	Eating factor[c]	Ruminating factor[d]	Total chewing factor[e]
Long			1.00	1.00	1.00
Coarse chopped	4.8 to 8.0		.95	.95	.95
Medium-coarse chopped	2.4 to 4.0		.90	.90	.90
Medium chopped	1.2 to 2.0		.85	.85	.85
Medium-fine chopped	.6 to 1.0		.85	.80	.80
Fine chopped	.3 to .5		.80	.65	.70
Ground or pelleted	.15 to .25		.40	.15	.25
Rolled			.80	.50	.60
Coarse ground or cracked		1.25	.60	.30	.40
Medium ground		.90	.50	.20	.30
Fine ground or pelleted		.63	.40	.15	.25

[a]Theoretical length of cut in cm.
[b]Grinder screen aperture in cm.
[c]Eating time (min/kg of NDF) equals the corresponding coefficient times 60.
[d]Ruminating time (min/kg of NDF) equals the corresponding coefficient times 110 - .8(FCM).
[e]Total chewing time (min/kg of NDF) equals the corresponding coefficient times 170 - .8(FCM).

Effect of Energy Supplementation of Alfalfa Hay or Alfalfa Silage on Protein Supply to Lactating Cows

D.B. Vagnoni and G.A. Broderick

Introduction

Ease of mechanization and reduced susceptibility to weather damage have made conservation of alfalfa as silage, rather than as hay, increasingly common. Substantial increases in milk and milk protein secretion from ruminally undegraded protein (RUP) supplementation in cows fed alfalfa silage (AS) diets indicated that intestinal protein supply was limiting. Moreover, response of milk protein secretion to RUP has been shown to be greater for AS than for alfalfa hay (AH) diets (Broderick 1995). Extensive conversion of protein to NPN occurring during silage fermentation results in excessive production of NH_3 in the rumen, suggesting that conservation of alfalfa as silage may reduce ruminal protein escape and (or) synthesis of ruminal microbial CP (MCP) relative to hay. Grinding high moisture corn (HMC) stimulated ruminal in vitro NH_3 uptake and milk protein secretion relative to no treatment of HMC. This suggests that enhancing the availability of ruminal fermentable energy may be an effective strategy for increasing microbial capture of ruminally degraded CP from AS. Measuring excretion of purine derivatives (PD) may provide a convenient method to estimate MCP supply in vivo because: 1) dietary purines are extensively degraded in the rumen and intestinal purines are almost exclusively of microbial origin, and 2) we previously reported (Vagnoni and Broderick 1995) a precise (r^2 = .93) linear relationship between PD excretion and intestinal purine flow. Therefore, we evaluated dietary responses of milk protein yield and MCP supply (estimated from PD excretion) to HMC supplementation of AH and AS diets. Specifically, we were interested in whether: 1) milk protein yield increased in response to HMC more on AS than AH diets and 2) whether dietary responses in milk protein yield corresponded to those observed for MCP.

Materials and Methods

Alfalfa silage was chopped to a theoretical length of 1.0 cm and ensiled in a concrete bunker silo at 41% DM. Alfalfa hay was wilted to approximately 85% DM, conserved as small rectangular bales and stored under shelter. Neither hay nor silage was rained on. Twelve multiparous Holstein cows were assigned to three replicated 4 x 4 Latin squares with 21-d periods. Days 1 to 14 of each period served as an adaptation period and all sample and data collection occurred during d 15 to 21. Diets were based on AH or AS and contained (DM basis) 24 or 40% HMC (Table 1). The HMC was ground through a 10-cm screen using a hammermill before mixing in the diet. Total urine collections were made using indwelling Foley catheters and urine output was measured for 3 days. Milk samples also were obtained at the a.m and p.m. milkings for the last 3 d of each experimental period. Ruminal samples were obtained at 0, 4, 8, and 12 h post-feeding for the determination of bacterial CP:purine ratio. Blood samples were taken 4 h after feeding on the last day of each period from the coccygeal artery or vein. Microbial CP flow was estimated using the following equation (Vagnoni and Broderick 1995):

$$\text{MCP, g / d} = \frac{\text{g MCP}}{\text{mmol purine}} \times \frac{\text{mmol /d PD excretion - 130}}{.856} \qquad (1)$$

Results and Discussion

Crude protein content was similar between AH and AS but fiber concentrations were higher in AH than in AS. Consequently, the fiber content was higher and the calculated NE_L content lower in AH than AS diets (Table 1). The high NPN content (56.9%) of AS was in accordance with typical observations in excess of 50%. Dry matter intake increased ($P <$ .05) in response to HMC level and to AH versus AS diets (Table 2). Yields of milk and milk protein also increased ($P <$ .001) in response to HMC level but were unaffected by forage source. Milk protein yield increased in response to HMC by 100 g/d and 170 g/d on AH and AS diets, respectively (Forage x HMC, P = .091). Assuming that the milk protein response to HMC was mediated principally through MCP supply, these data suggest that the protein

status of cows consuming AS diets was poorer (and hence more responsive) than protein status of cows consuming AH diets. Total PD excretion and urinary allantoin:creatinine were increased ($P < .05$) by both increasing HMC level and AH versus AS diets and responded more to HMC on AS than AH diets (i.e., Forage x HMC interaction was significant). Plasma allantoin concentrations were increased in response to HMC level ($P < .01$), and the response was greater on AS than AH diets (Forage x HMC, P = .081). The bacterial CP:purine ratio decreased (P =.01) in response to HMC level and was lower for AH than AS diets ($P < .001$). This ratio is important because it directly influences the calculation of MCP supply (Eqn. 1 above). Estimated MCP supply was increased by HMC level ($P < .001$), more so on AS than on AH diets ($P = .022$); there was no effect due to forage source.

Conclusions

These results indicated that availability of ruminal degradable CP, relative to energy, for MCP synthesis was in excess on AS compared to AH diets. Also, the nature of the response to supplementation of HMC paralleled that for milk protein yield and suggested that PD excretion accurately predicted the response of MCP yield to the diet.

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Table 1. Composition of diets[1].

Item	AH	AS	AH + HMC	AS+ HMC
	% of DM			
AH	75.0	...	55.0	...
AS	...	75.0	...	55.0
Ground HMC	24.0	24.0	40.0	40.0
Soybean meal	...	...	3.5	3.5
Sodium bicarbonate	...	...	.5	.5
Sodium phosphate	.5	.5	...	...
Dicalcium phosphate	...	...	.5	.5
TMS & vitamins[2]	.5	.5	.5	.5
Chemical composition				
CP	17.0	17.3	16.2	16.4
NDF	35.6	33.0	30.4	28.5
ADF	25.0	23.8	19.9	19.1
NE_L, Mcal/kg DM	1.54	1.60	1.65	1.69

[1]AH = alfalfa hay, AS = alfalfa silage, HMC = high moisture corn, TMS = trace mineralized salt.

Table 2. DM intake, milk yield, purine derivatives, and microbial crude protein supply.

Item	AH	AS	AH+HMC	AS+HMC	SE	$P > F^2$ Forage	HMC	FxHMC
DMI, kg/d	22.8	21.9	24.2	23.5	.4	.044	<.001	.784
Milk, kg/d	29.6	28.2	31.6	31.8	.7	.351	<.001	.262
Milk protein, kg/d	.96	.90	1.06	1.07	.02	.219	<.001	.091
PD excretion, mmol/d	552	473	603	568	11	<.001	<.001	.049
Urinary A:C	3.23	2.75	3.43	3.18	.05	<.001	<.001	.021
Plasma A:C	41.6	39.6	43.2	46.4	1.4	.661	.006	.081
MCP:purine, g/mmol	.604	.713	.570	.666	.001	<.001	.010	.553
MCP, g/d	1981	1925	2081	2262	50	.222	<.001	.022

[1]AH = Alfalfa hay, AS = alfalfa silage, HMC = high moisture corn.
[2]Probability of a significant contrast effect, FxHMC = Forage x HMC interaction.

Effect of Grinding High Moisture Corn on Its Utilization by Dairy Cows Fed Alfalfa Silage

C. Ekinci and G.A. Broderick

Introduction

Alfalfa silage (AS) is one of the forages most commonly fed to dairy cows. However, during ensiling 50 to 60% of its CP is converted to NPN. Synchronization of energy fermentation and CP degradation in the rumen may improve the efficiency of utilization of the NPN for microbial protein synthesis (MPS) and reduce the need for dietary RUP. Because of the structure of its starch granule (McAllister et al. 1993), corn is not extensively degraded in the rumen. Processing of corn improves its digestibility in the rumen and the intestine (Owens et al. 1986). Ensiling of high moisture corn is one of the methods used to increase corn digestibility (Hale 1973). Grinding of ensiled high moisture corn may have an additive effect on digestibility. Our objective was to determine if grinding high moisture corn would improve its utilization in lactating dairy cows by increasing ruminal MPS and reducing the need for RUP. Therefore, milk yield should increase, and response to RUP decrease, with the feeding of ground high moisture corn.

Materials and Methods

Fifty-two (52) Holstein cows (36 multiparous, 8 with ruminal cannulae and 16 primiparous) with mean (± SD) 35.8 (± 6.1) kg/d milk yield and 110 (± 39 d) DMI were randomly assigned to one of four diets during a 12-week lactation study. The main concentrate was either 68% DM high moisture ear corn (HMC), rolled before ensiling, or the same HMC that was ground (GHMC) through a 9.5 mm (3/8″) screen as it came out of the silo. Expeller soybean meal (ESBM) was added as a source of RUP to two of the diets. The four diets contained (DM basis): 1) 53% AS plus 42% HMC (HMC); 2) 53% AS plus 30% HMC plus 12% ESBM (HMC + E); 3) 53% AS plus 42% GHMC (GHMC); 4) 53% AS plus 30% GHMC plus 12% ESBM (GHMC + E). Diets contained either 16.3% CP (HMC and GHMC) or 21.3% CP (HMC + E and GHMC + E); NE_L content of all diets was 1.69 Mcal/kg. The NDF contents were 26% for the HMC + E and GHMC + E diets and 28% for the HMC and GHMC diets. Ration composition, yield of milk and milk components, ruminal pH and ammonia, blood and milk urea, and DM and starch digestion, estimated using indigestible ADF as an internal marker, were determined during the trial. Stimulation of MPS by HMC and GHMC was assessed from ammonia disappearance in duplicate in vitro incubations.

Results and Discussion

Changes in ammonia concentration in ruminal in vitro incubations conducted with HMC and GHMC are shown in Figure 1. Grinding of HMC significantly ($P < .01$) decreased ammonia at 3, 4, 5 and 6 h of incubation, presumably by stimulating ruminal MPS. The results from the in vitro incubations reflected those observed in the lactation trial, which are summarized in Table 1. There were no differences in BW gain despite DMI being lower on diet HMC. Grinding of HMC significantly increased digestibility of DM and starch. The higher DMI and improved digestibility were reflected in improved yield of milk and milk components, particularly on diet GHMC; grinding of HMC resulted in equivalent production between diets HMC + E and GHMC. Although there was no difference in milk yield among the HMC + E, GHMC and GHMC + E diets, yields of fat, protein, and SNF were lower on diet GHMC + E than on diets HMC + E and GHMC. This result may have been related to hot weather occurring during the middle four weeks of the 12-week trial and not due to an adverse effect of feeding RUP. During the first four weeks of the trial, cows fed the GHMC + E diet produced more milk than those fed the GHMC diet but, after a four-week heat wave starting in week 5, cows receiving the GHMC + E diet began to decline. This may have resulted from greater incidence of sub-clinical mastitis in cows fed the GHMC + E diet because, by chance, the cows assigned to this diet had higher SCC before the trial. Moreover, their SCC increased more during the trial. As a result, the main effects of

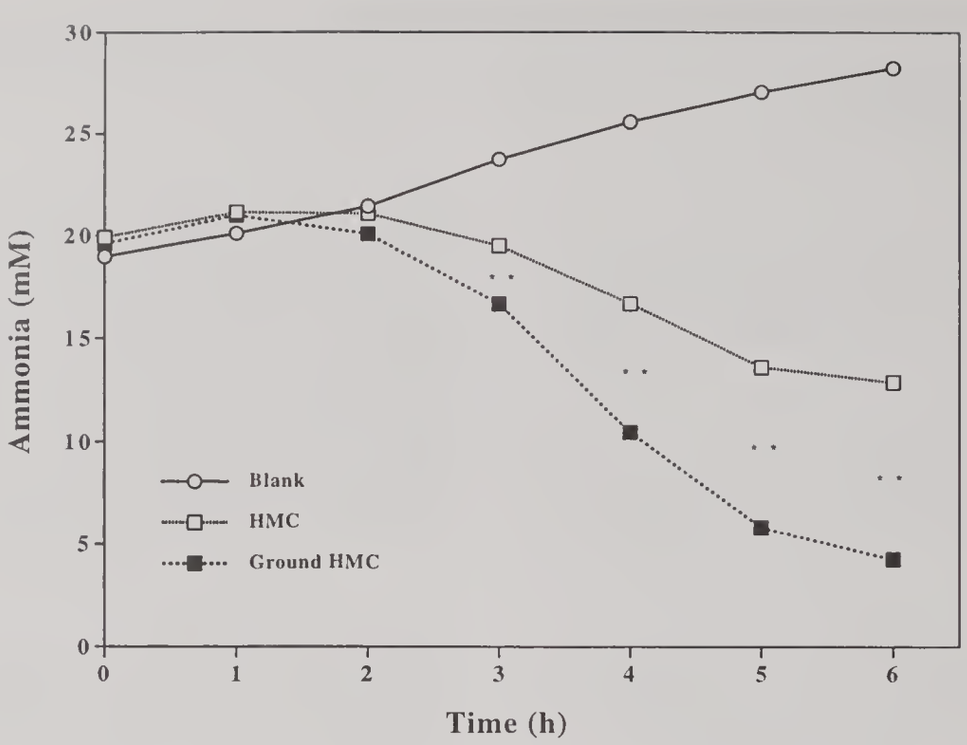

*Figure 1. In vitro ruminal ammonia concentration in 6-h incubations containing no added substrate (Blank), high moisture corn (HMC) or ground HMC (** P < .01).*

ESBM and grinding cannot be seen clearly because the two factors interacted ($P = .06$). Efficiency of milk yield (weekly milk yield divided by weekly DMI) was not different among the treatments. However, N efficiency was higher for the HMC and GHMC diets than for the two diets with added ESBM; the GHMC + E diet had the lowest N efficiency. Persistency of milk yield tended to follow differences in milk yield and was lower for the HMC diet than for the other three diets. Concentration of urea in milk mirrored that in blood plasma; both were higher for diets containing ESBM. Elevated blood and milk urea observed with ESBM feeding probably resulted from inefficient use of excess dietary N at both the rumen and tissue level. Although ruminal pH was not significantly influenced by diet, grinding of HMC decreased ammonia concentration ($P < .01$). This suggested greater ruminal fermentation with the GHMC diet, compared to the HMC diet, increased ammonia utilization and MPS by the ruminal microbes. Adding ESBM to the diets increased ruminal ammonia.

Conclusions

Grinding of HMC improved milk yield 2.4 kg/d; cows fed the GHMC diet also had significantly greater yields of fat, protein, lactose, and SNF than cows fed the HMC diet. Milk yield of cows fed the GHMC + E diet was 2 kg/d lower than cows fed the HMC + E diet; this may be related to the higher SCC present in cows fed the GHMC + E diet. Grinding of HMC increased apparent total tract digestibility of DM and starch and decreased ruminal ammonia concentration. Greater ruminal fermentation of ground HMC, versus unground HMC, probably improved utilization of NPN from AS by increasing MPS. Grinding of HMC improved its utilization in the lactating dairy cow.

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Table 1. Effects of grinding high moisture corn on DMI and BW gain, apparent DM and starch digestion, yield of milk and milk components, blood and milk urea, and ruminal pH and ammonia.[1]

Item	HMC	HMC + E	GHMC	GHMC + E	SEM	$P > F^2$
DMI, kg/d	21.2[b]	22.6[ab]	23.1[a]	22.2[ab]	.5	0.064
BW change, kg/d	.15	.09	.09	.18	.09	0.86
DM digestibility, %	66.1[b]	64.8[b]	69.7[a]	69.3[a]	1.1	<.01
Starch digestibility, %	94.5[b]	93.8[b]	98.7[a]	98.8[a]	.5	<.01
Milk yield, kg/d	30.1[b]	34.0[a]	32.5[ab]	32.1[ab]	.9	0.03
Fat, %	3.58[ab]	3.45[b]	3.75[a]	3.35[b]	.10	0.04
Fat, kg/d	1.07[b]	1.18[a]	1.21[a]	1.02[b]	.03	< .01
Protein, %	3.03[ab]	2.95[b]	3.17[a]	2.99[b]	.06	0.09
Protein, kg/d	.91[b]	1.01[a]	1.03[a]	.91[b]	.02	< .01
Lactose, %	4.71[b]	4.92[a]	4.93[a]	4.98[a]	.06	< .01
Lactose, kg/d	1.42[c]	1.70[a]	1.62[ab]	1.53[bc]	.04	< .01
SNF, %	8.43[b]	8.55[ab]	8.79[a]	8.66[ab]	.12	< .01
SNF, kg/d	2.53[b]	2.95[a]	2.88[a]	2.65[b]	.07	< .01
Efficiency[3]	1.43	1.52	1.42	1.46	.04	0.24
N efficiency[4], %	25.5[a]	20.4[b]	26.7[a]	18.8[c]	.5	< .01
Persistency[5]	.83[b]	.93[a]	.92[a]	.89[ab]	.02	0.01
Blood urea, mg N/dL	13.0[c]	21.9[a]	12.2[c]	19.0[b]	.5	<.01
Milk urea, mg N/dL	11.8[c]	21.0[a]	11.0[c]	18.6[b]	.5	<.01
Ruminal pH	6.24	6.07	6.04	6.09	.06	0.31
Ruminal ammonia, mM	10.1[b]	13.6[a]	6.9[c]	11.5[ab]	.6	<.01

[a,b,c]Means within the same row without a common superscript differ ($P < .05$).

[1]HMC = High moisture corn diet; HMC + E = High moisture corn plus expeller soybean meal diet; GHMC = Ground high moisture corn diet; GHMC + E = Ground high moisture corn plus expeller soybean meal diet; SEM = Standard error of the mean.

[2]Probability of a significant effect of diet.

[3]Milk yield / DMI.

[4]Milk N / N intake x 100.

[5]Pre-experiment milk yield / Experiment milk yield.

Use of Milk Urea as an Indicator of Nitrogen Utilization in the Lactating Dairy Cow

G.A. Broderick

Introduction

Urea is the primary form in which N is excreted in mammals; therefore, elevated blood urea is related to inefficient utilization of dietary CP. Concentrations of milk and blood urea are highly correlated (Oltner and Wiktorsson 1983, Gustafsson and Palmquist 1993) and milk urea N (MUN) serves as an easily sampled indicator of blood urea N (BUN). Milk urea N has attracted attention as a possible index of excessive ruminal protein degradation. Hutjens and Barmore (1995) suggested MUN in the range of 12 to 17 mg N/dL would indicate optimal balance of RDP and ruminally fermentable energy. Since 1978, we have measured MUN in feeding trials with lactating cows. The objective here was to use these data to study the relationship of MUN to a number of variables related to diet, protein yield and N efficiency.

Materials and Methods

Concentrations of MUN were measured in 22 feeding trials using 419 lactating cows fed 78 different diets; these were conventional production trials in which intakes of DM, CP and estimated NE_L, change of BW, BUN concentrations, and production of milk, fat, protein and lactose were determined. In 16 of the trials, 76 ruminally cannulated cows were used in switch-back arrangements of treatments to quantify ruminal ammonia on 56 of these diets. In 10 of the trials, fecal grab samples were taken from 203 lactating dairy cows fed 38 diets and internal markers (either acid insoluble ash or indigestible ADF) used to determine digestible DM and to compute NE_L assuming digestible DM was equivalent to TDN. Concentrations of MUN and BUN were determined in all trials using a diacetyl monoxime colorimetric assay adapted to Technicon AutoAnalyzers. All of these data already are published in seven papers and two theses. Linear regression of MUN on each variable studied was used to assess the relationships.

Results and Discussion

Table 1 lists 16 equations obtained from linear regression of MUN level (mg N/dL) and, in one case, MUN secretion rate (mg N/d) on nine different variables. Regressions for the eight variables that were derived from the complete data set (equations 1 through 8) were significant ($P >$ .001). The highest correlation was obtained from regressing MUN on BUN (equation 1; $r^2 = .743$); Gonda and Lindberg (1994) reported a correlation coefficient of .73 for MUN and BUN. The strong relationship between MUN and BUN is illustrated in Figure 1. An interesting finding was that MUN was not nearly as well correlated to ruminal ammonia concentration (equation 9; $r^2 = .429$; Fig. 2) as it was to a number of factors related to dietary CP and N efficiency. Milk urea N was more highly correlated to dietary CP content (equation 2; $r^2 =$ .588) than to CP intake (equation 5.1; $r^2 = .439$). Amount of MUN secreted (mg N/d) was more poorly correlated than MUN concentration for every variable except for CP intake (equation 5.2; $r^2 = .601$). Concentration of MUN was correlated similarly to N efficiency (milk N:N intake; equation 7) and to excess N intake (N intake minus milk N; equation 8); both explained about 55% of the variation in MUN concentration. These results indicated that MUN may be more useful as an index of overall N inefficiency than as an index of excessive ruminal protein degradation. Solving

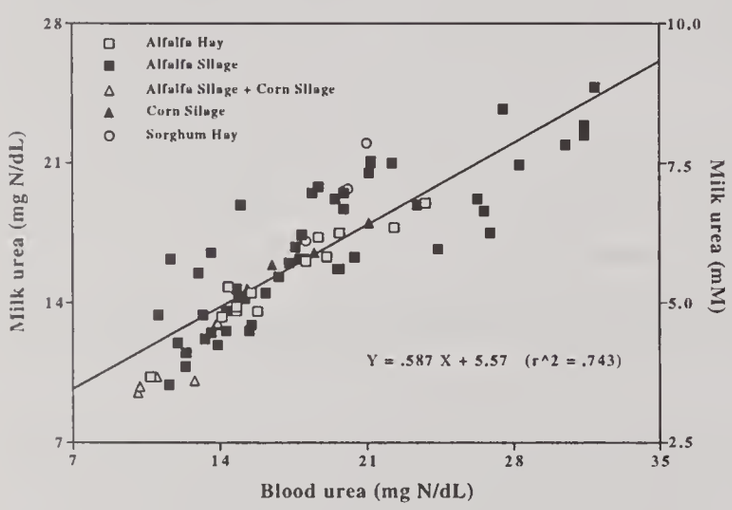

Figure 1. Regression of milk urea N concentration on blood urea N concentrations observed in 419 lactating dairy cows fed 78 diets in 22 different trials.

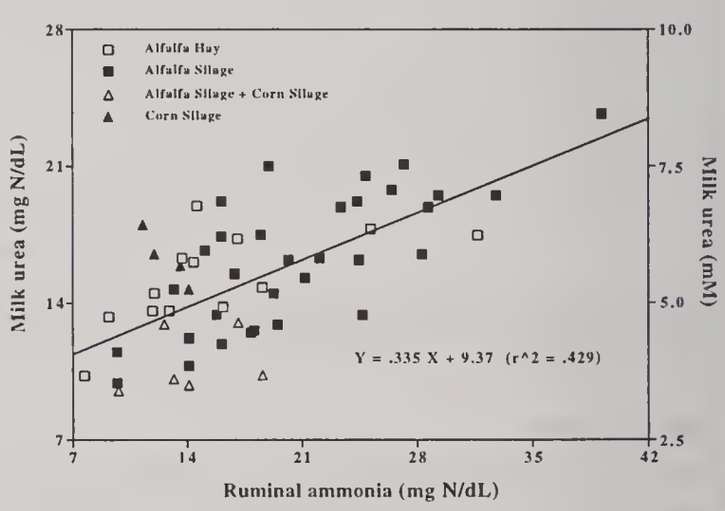

Figure 2. Regression of milk urea N concentration on ruminal ammonia concentrations observed in 76 ruminally cannulated lactating dairy cows fed 56 diets in 16 different trials.

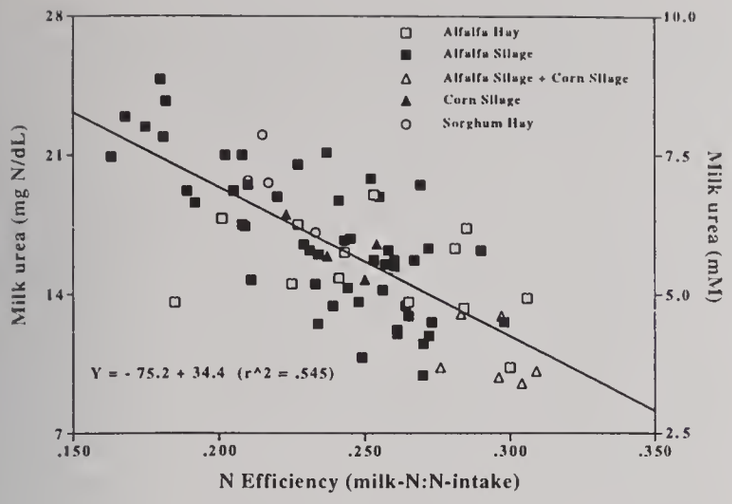

Figure 3. Regression of milk urea N concentration on N efficiencies (milk N:N intake) observed in 419 lactating dairy cows fed 78 diets in 22 different trials.

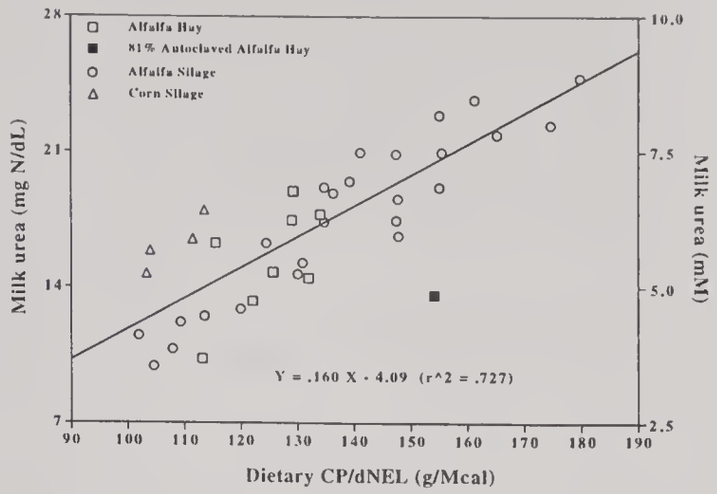

Figure 4. Regression of milk urea N concentration on the ratios CP/dNE$_L$ (computed from digestible DM) observed in 203 lactating dairy cows fed 38 diets in 10 different trials.

regression equation 7 (Fig. 3) for the MUN corresponding to the average N efficiency from all 78 diets (milk-N:N-Intake = .243) yielded a MUN estimate of 16.1 mg N/dL; MUN greater than this would imply a N efficiency of less than 24%.

Using data from all 78 diets, correlation of MUN to dietary content and intake of NE$_L$, computed from NRC tables (nNE$_L$, equations 3 and 4), was not as strong as it was to the variables derived from dietary CP. The ratio, dietary CP/nNE$_L$, also was not as well related to MUN as was dietary CP alone. Dietary NE$_L$ content and intake also were estimated from digestible DM (dNE$_L$) on 38 of the diets. On these diets, MUN was not significantly correlated ($P > .06$) to intake of either nNE$_L$ or dNE$_L$ (equations 10 and 11). However, CP/dNE$_L$ was more highly correlated to MUN (equation 13.1; $r^2 = .650$) than was CP/nNE$_L$ (equation 12.1; $r^2 = .564$). Inspecting the regression of MUN on CP/dNE$_L$ (Fig. 4) suggested that data from one diet, which contained 81% autoclaved alfalfa hay, was an outlier; deleting this point substantially improved the fit of this regression (equation 13.2; $r^2 = .727$) as well as the regression of MUN on CP/nNE$_L$ (equation 12.2; $r^2 = .654$).

Conclusions

Concentration of MUN was highly correlated to BUN and dietary CP/dNE$_L$ (NE$_L$ estimated from digestible DM). Milk urea N was more highly correlated to N efficiency than to ruminal ammonia concentration; MUN explained 55% of the variation in N efficiency in this data set. Scatter of data for the regression of MUN on N efficiency was similar for efficiencies ranging from 16 to 30%. Although MUN concentration will be a useful index of CP utilization, specific MUN values are only approximate guidelines to the protein status of the lactating cow.

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Table 1. Linear regression equations.[1]

X-variable	Eq. no.	Equation	n	r^2	$P > F^2$
Blood urea N, mg N/dL	1	Y1 = .587 X + 5.57	78	.743	< .001
Dietary CP, % of DM	2	Y1 = 1.37 X - 8.48	78	.588	< .001
Dietary nNE_L, Mcal/kg DM	3	Y1 = - 16.8 X + 42.9	78	.198	< .001
Dietary CP/nNE_L, g/Mcal	4	Y1 = .147 X - .558	78	.529	< .001
CP intake, kg/d	5.1	Y1 = 4.74 X - 3.03	78	.439	< .001
	5.2	Y2 = 188 X - 250	78	.601	< .001
nNE_L intake, Mcal/d	6	Y1 = - .216 X + 23.9	78	.058	.033
N efficiency (milk N:N intake)	7	Y1 = - 75.2 X + 34.4	78	.545	< .001
Excess N intake (N intake - Milk N), g/d	8	Y1 = .0342 X - .696	78	.552	< .001
Ruminal NH_3, mg N/dL	9	Y1 = .335 X + 9.37	56	.429	< .001
Dietary nNE_L, Mcal/kg DM	10	Y1 = -3.59 + 11.7	38	.093	.063
Dietary dNE_L, Mcal/kg DM	11	Y1 = - 3.14 X + 10.5	38	.046	.084
Dietary CP/nNE_L, kg/Mcal	12.1	Y1 = .135 X + .700	38	.564	< .001
	12.2[2]	Y1 = .147 X - .559	37	.654	< .001
Dietary CP/dNE_L, g/Mcal	13.1	Y1 = .150 X - 3.03	38	.650	< .001
	13.2[2]	Y1 = .160 X - 4.09	37	.727	< .001

[1]Y1 = Milk urea N Concentration (mg N/dL); Y2 = milk urea N secretion rate (mg N/d); nNE_L = NE_L computed from ration composition using NRC tables; dNE_L = NE_L computed from ration digestible DM determined using fecal marker ratio techniques, assuming digestible DM = TDN; n = number of diets used in the linear regression.

[2]Regressions obtained after deleting data from one diet that contained 81% of DM as autoclaved alfalfa hay.

Excretion of Purine Derivatives by Dairy Cows Abomasally Infused With Incremental Amounts of Purines

D.B. Vagnoni and G.A. Broderick

Introduction

Standard in vivo methods of estimating ruminal microbial protein supply require using exogenous markers (e.g., ^{15}N-ammonium salts) and animals that are cannulated in the abomasum or small intestine. Cannulations of this type are undesirable from an animal health standpoint; furthermore, estimation of microbial protein flow using markers and animals cannulated in the lower tract is laborious and imprecise. Topps and Elliott (1965) reported a correlation between urinary allantoin excretion and purine concentration in the rumen of sheep, suggesting the utility of allantoin excretion as an index of ruminal microbial protein flow. Subsequently, quantitative relationships between urinary purine derivative (PD) excretion and purine flow rates have been reported in sheep and cattle maintained by total intragastric nutrition. Because extrapolation of these data to lactating dairy cows seemed dubious, we determined the relationship between intestinal purine flow and urinary PD excretion using Holstein cows, maintained in a normal nutritional state, with body weight and purine flows approximating that of lactating cows.

Materials And Methods

Five ruminally cannulated Holstein cows (two lactating, three dry) were fed a diet consisting of corn silage (37.5% DM) plus dicalcium phosphate (190 g/d). Cows had free access to water and trace-mineralized salt blocks throughout the trial. Lactating cows were milked twice daily and were

dried off immediately following completion of period 5. Purines were infused at five equally spaced levels as brewers yeast (Labudde Feed Co., Grafton, WI) during five experimental periods (P2 to P6) according to a Latin square design. Purine flow arising from the normal ruminal microbial population was determined in all cows during the two periods immediately preceding (P1) and following (P7) the infusion periods, as well as in each cow receiving 0 g/d purines during the infusion periods (i.e., P2 to P6). All experimental periods were 7 d in length. Suspensions of brewers yeast were continually mixed on magnetic stirrers and infused, using a multi-channel peristaltic pump, via tubing passed through the rumen cannula, through the rumen and omasum, and into the abomasum. Infusions were initiated at 1800 h on d 3 of each experimental period and maintained for 96 h. Total urine collections were made using indwelling Foley catheters which were inserted 24 h following initiation of infusions and daily urine output was measured for three days. Fresh containers with 500 ml of 1.5 N H_2SO_4 were attached to each cow at 0700 and 1800 h of each d. Purine derivatives (allantoin plus uric acid) were determined in milk and urine samples and creatinine was determined in urine samples.

Results and Discussion

The relationship between total PD excretion (mmol/d) and purine flow (mmol/d) was described by the equation:

PD excretion = .856 x purine flow + 103 (r^2 = .93) (1)

In addition to total PD excretion, both allantoin excretion (r^2 = .94) and the urinary allantoin:creatinine ratio (r^2 = .88) were highly correlated with purine flow (Fig. 1). By measuring the microbial crude protein (MCP) to purine ratio of ruminal microorganisms and rearranging Eqn. 1, MCP flow can be estimated from PD excretion using the equation:

$$\text{MCP, g / d} = \frac{\text{g MCP}}{\text{mmol purine}} \times \frac{\text{mmol /d PD excretion - 130}}{.856} \qquad (2)$$

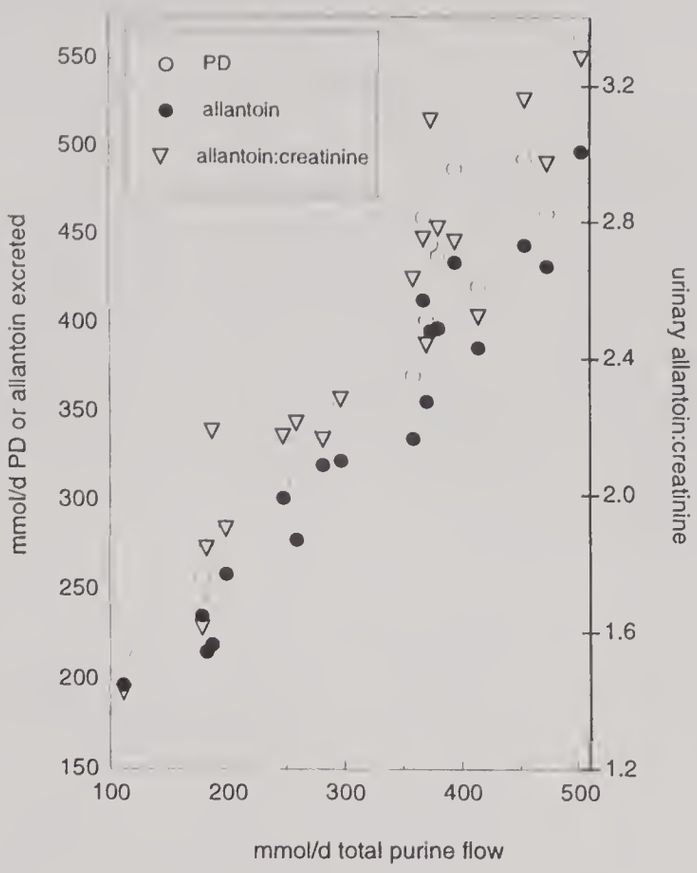

Figure 1. Relationship of total purine derivative (PD) excretion, allantoin excretion, and urinary allantoin:creatinine ratio with abomasal purine flow.

Conclusion

Purine derivative excretion responded linearly to abomasal purine infusions in Holstein cows. Combined with a measurement of the CP to purine ratio in ruminal microbes, measurement of PD excretion offers promise as a useful method to estimate microbial crude protein supply in vivo.

Reference

Topps, J.H. and R.C. Elliott. 1965. Relationships between concentrations of ruminal nucleic acid and excretion of purine derivatives by sheep. Nature 205:498-500.

Feeding High Oil Corn to Lactating Dairy Cows

T.R. Dhiman, B. Hoogendijk, R.P. Walgenbach and L.D. Satter

Introduction

Fats and oils are used in diets of high producing dairy cows to increase energy density. Use of feed ingredients that are normally high in oil can be a less expensive way to add fat to the diet than by adding tallow. High oil corn could be one of these sources. The objective of this study was to quantify the effect of additional fat supplied by high oil corn compared with normal corn on dairy cow performance.

Materials and Methods

Fifty-three dairy cows (31 multiparous and 22 primiparous cows) were assigned randomly before calving to normal corn (NC) and high oil corn (HOC) treatments. A pretrial diet was fed during the first two wks of lactation. Starting at wk 3 and lasting through wk 24 of lactation, cows were fed diets containing either normal high moisture ear corn and corn silage (NC) or high oil high moisture ear corn and corn silage (HOC). High oil corn (mixture of Hybrid-5501 and 4401; Cenex Land O'Lakes) and a control variety of comparable maturity (Hybrid LOL-522; Cenex Land O'Lakes) were used as high moisture ear corn. High oil corn (Hybrid 671 EDPP) and control corn (Dekalb DK-512) of comparable maturity were used as corn silage. Both types of corn were grown at the US Dairy Forage Research Center Farm Facility, Prairie du Sac, WI during 1994 for use in this experiment. Chemical composition of diet ingredients is in Table 1. Ingredient composition of diets is in Table 2. Daily feed intake and milk yield were recorded. Weekly milk samples were analyzed for composition. Cows were weighed weekly.

Results

Cows fed diets containing normal or high oil corn had similar feed intake (Table 3). Milk yield, milk fat and lactose contents were unaffected by the corn treatments. Milk protein contents were slightly lower with the high oil corn treatment. Cows fed normal or high oil corn had similar body weight gain.

Summary

The high oil corn hybrids used in this study to supply both corn grain and corn silage did not improve lactation performance of dairy cows.

Table 1. Chemical composition of diet ingredients.

Item	DM %	CP	NDF	ADF	Fatty acids[1]
			% DM basis		
Alfalfa silage	37.3	20.9	43.0	34.6	1.74
Normal corn silage	36.6	7.2	42.4	24.0	2.12
High oil corn silage	36.8	7.9	40.4	23.4	3.02
Normal high moisture ear corn	69.0	8.8	13.3	3.05	3.96
High oil high moisture ear corn	75.0	8.9	13.8	2.90	6.32

[1]Sum of C14:0 to C18:3.

Table 2. Ingredient composition of diets and chemical composition of total diet.

Ingredient	Pretrial	Treatment NC	HOC
Alfalfa silage	15.5	27.5	27.5
Chopped alfalfa hay	12.0	-	-
Control corn silage	11.25	22.5	-
High oil corn silage	11.25	-	22.5
Control high moisture ear corn	15.75	31.5	-
High oil high moisture ear corn	15.75	-	31.5
Soybean meal	10.0	10.0	10.0
Roasted soybeans	4.6	4.6	4.6
Blood meal	2.0	2.0	2.0
Sodium bicarbonate	.2	.2	.2
Dicalcium phosphate	1.0	1.0	1.0
Trace-mineralized salt	.7	.7	.7
Vitamin ADE[1]	trace	trace	trace
Total mixed ration DM, %	54.6	49.6	50.6
NE , Mcal / kg DM	1.671	1.657	1.685
CP, % DM	18.8	19.1	19.3
Fatty acids[2], % DM	3.91	3.43	4.38

[1]Vitamin A, 148,500; Vitamin D, 49,500; and Vitamin E, 495 IU/d per cow.
[2]Sum of C14:0 to C18:3.

Table 3. Lactation performance.

Measurement	Treatment NC	HOC	SEM	*P*
Feed intake, kg/d	21.7	21.6	.4	.6
Milk yield, kg/d	36.5	36.0	.8	.7
3.5% FCM, kg/d	35.4	34.8	.7	.6
Milk fat, %	3.39	3.28	.05	.14
Milk protein, %	3.00	2.93	.03	.1
Milk fat yield, kg/d	1.22	1.18	.03	.4
Milk protein yield, kg/d	1.07	1.07	.02	.9
Lactose, %	4.95	4.91	.03	.4
FCM / kg feed intake	1.63	1.67	.02	.2
Body weight (BW), kg				
Beginning (BW at wk 2)	577	558		
End (BW at wk 23)	607	586		
Gain (End BW minus beginning)	29.7	28.0		

Particle Size of Roasted Soybeans and Its Effect on Milk Production of Dairy Cows

T.R. Dhiman, A.C. Korevaar and L.D. Satter

Introduction

Heat treatment of soybeans increases the amount of rumen undegraded protein, and use of roasted soybeans as a dietary supplement for dairy cows has gained wide spread acceptance. A question remains, however, as to optimum particle size for the roasted soybeans. The protein in small soybean particles is likely to be degraded more rapidly than the protein in large particles. Therefore, the objective of this experiment was to quantify the effect of particle size of roasted soybeans on milk production of high producing dairy cows.

Materials and Methods

Fifteen multiparous cows were used in a 5 x 5 Latin square design experiment with a 2 wk pretrial and five experimental periods of 2 wks each. At the end of the pretrial period, cows were blocked according to their milk yield and randomly assigned to one of the five treatments. The first week in each period was used as adaptation time, and measurements were made during the last week in each period. At the beginning of the experimental period, cows were between 126 to 171 days in milk. The diet contained (% of DM): alfalfa silage, 33.0; corn silage, 17.0; high moisture ear corn, 30.6; soybeans, 18.0; and mineral and vitamin supplement, 1.4. The five treatments had soybeans in the diet as: raw whole soybeans (RAWSB), and roasted soybeans in four different particle sizes [whole and half (RSBWH), half and quarter (RSBHQ), quarter and smaller (RSBQ), and coarsely ground soybeans (RSBG)]. Roasted soybeans of different particle size were prepared by passing through a roller mill and then screening to different sizes. Particle size distribution of soybeans is given in Table 1. Soybeans were roasted at 146°C using a Jet Pro Roaster and steeped for 30 min before cooling.

Diets were fed as a total mixed ration once daily. Daily feed offered and refused was recorded. Cows in all the treatments were injected with rBST biweekly. During the last two days in each period, milk samples were collected from four consecutive a.m. and p.m. milkings and analyzed for composition. Feed DM digestibility was determined using acid detergent lignin as an internal marker. During the last week in each period, six fecal grab samples were collected within 48 h from two cows in each treatment. Composite fecal samples of each cow during each period were analyzed for acid detergent lignin. During the last week in each period, fecal grab samples (500 g on fresh basis) from each cow were washed with water through different screens for visible soybean particles. Soybean particles were dried at 60°C and expressed as g soybean particles excreted/ 100 g of dried feces.

Results

Feed intake did not differ among treatments. Cows fed RSBWH and RSBHQ produced more 3.5% FCM compared with RAWSB, RSBQ, and RSBG (Table 2). Milk yield of cows fed RSBWH and RSBHQ was not different. Normal handling of properly roasted soybeans will result in a large number of seeds being broken into halves (36% on w/w basis in the present experiment). Milk fat, protein, lactose, and SNF percent were similar across treatments. Higher milk yield of cows fed RSBWH and RSBHQ resulted in increased milk fat and protein yield compared with other treatments. Feed DM digestibility was similar in all soybean treatments. Cows fed RAWSB excreted the highest amount of soybeans in the feces, whereas cows fed RSBG excreted the least amount of soybeans in the feces.

Conclusion

Results suggest that the benefit of reducing the particle size of properly roasted soybeans beyond wholes and halves was minimum. Grinding of roasted soybeans is definitely not recommended.

Table 1. Particle size distribution of different soybean treatments.

Treatment[1]	Screen size, mm 4.75	3.35	2.36	1.18	Pan
	% retained on the screen				
RAWSB	92.6	6.1	.9	.3	.07
RSBWH	90.6	8.0	.8	.3	.3
RSBHQ	21.2	36.9	23.9	10.7	7.3
RSBQ	30.5	21.1	17.7	16.7	14.0
RSBG	5.1	10.4	23.5	32.1	28.9

[1]Five treatments had soybeans in the diet as raw whole soybeans (RAWSB), and roasted soybeans in four different particle sizes [whole and half (RSBWH), half and quarter (RSBHQ), quarter and smaller (RSBQ), and coarsely ground soybeans (RSBG)].

Table 2. Dry matter intake, milk production, and feed digestibility of cows fed soybeans of different particle sizes.

Measurement	Treatment[1] RAWSB	RSBWH	RSBHQ	RSBQ	RSBG	SEM	*P* =
DMI, kg/d	24.3	24.0	23.7	23.6	23.9	.6	.9
Milk, kg/d	36.2^{c}	38.1^{ab}	38.7^{a}	37.4^{ac}	37.0^{bc}	.5	.03
3.5% FCM[2], kg/d	35.4^{b}	37.7^{a}	37.2^{ab}	35.1^{b}	35.4^{b}	.8	.08
Milk fat, %	3.37	3.43	3.27	3.16	3.25	.08	.2
Milk protein, %	3.04	3.04	3.02	3.04	3.08	.03	.6
Fat yield, kg/d	1.22^{ab}	1.31^{a}	1.27^{ab}	1.17^{b}	1.20^{b}	.04	.1
Protein yield, kg/d	1.10^{b}	1.15^{a}	1.17^{a}	1.13^{ab}	1.14^{ab}	.02	.08
Feed DM digestibility, %	63.6	64.0	64.6	64.3	64.0	1.5	.5
Soybean excretion, g/100g feces	6.13^{a}	3.10^{b}	3.34^{b}	2.27^{bc}	1.06^{c}	.5	.001

[abc]Means in the same row with different superscript differ, *P* as indicated.

[1]Five treatments had soybeans in the diet as raw whole soybeans (RAWSB), and roasted soybeans in four different particle sizes [whole and half (RSBWH), half and quarter (RSBHQ), quarter and smaller (RSBQ), and coarsely ground soybeans (RSBG)].

[2]3.5% FCM = .432(kg milk) + 16.2 (kg fat).

Feeding Heat Treated Cottonseed to Lactating Dairy Cows

T.R. Dhiman and L.D. Satter

Introduction

Protein supplements with high energy content that are resistant to microbial protein degradation in the rumen, yet available for absorption in the small intestine, may help supply the extra energy and protein needed for high milk production during early lactation. Linted-cottonseed on a dry basis contains approximately 20% fat and 20% crude protein and can be used effectively as a fat, protein and fiber supplement. Heat treatment has been used as a safe and economical method to reduce protein degradation by rumen microbes. Chemical, in vitro and in situ evaluation of linted-cottonseed exposed to different heat treatments indicates that heat treatment of cottonseed should increase the supply of protein to the small intestine.

The objective of this experiment was to compare optimally heat treated linted-cottonseed with solvent extracted soybean meal and unheated linted-cottonseed as a protein supplement for lactating dairy cows.

Materials and Methods

Linted-cottonseeds were brought to 146°C in a Jet Pro roaster and steeped for 30 min prior to cooling. Fifty-one multiparous cows were blocked according to milk yield in the previous lactation. Cows within each block were randomly assigned to one of three treatments according to expected calving date. The three treatments were soybean meal (SBM), linted-cottonseed (LCS), and heat treated linted-cottonseed (HLCS). Treatment diets were fed through wk 28 of lactation. Diets were fed as total mixed rations once daily. Ingredient composition of the diets is in Table 1. Daily feed offered and refused was recorded. Milk weights were recorded twice daily. Weekly milk samples were analyzed for composition. Cows were weighed weekly and scored once a month for body condition. Cows in all treatments received biweekly injection of rBST starting at week 9 of lactation.

Results

Results are summarized in Table 2. Cows in the SBM, LCS, or HLCS treatments had similar feed intake. Cows fed HLCS produced 1.9 kg more 3.5% FCM compared with cows in the SBM or LCS treatments ($P = .3$). Similar feed intake and a slight increase in milk yield increased the feed efficiency of cows fed HLCS ($P = .1$). Milk fat percent was the same across treatments. Milk protein content was decreased in cows fed cottonseed compared with soybean meal. Cows in the HLCS treatment started the experiment with lower milk protein content. During the previous lactation, milk protein content of cows in the SBM, LCS, and HLCS treatments was 3.31, 3.31, and 3.22, respectively, suggesting the two cottonseed treatments probably did not differ in milk protein content. Cows fed cottonseed (heated or unheated) had slightly higher body weight gain and body condition score.

Summary

Results suggest that feeding heat treated linted cottonseed improved dairy cow performance.

Table 1. Ingredient and chemical composition of diets.

	Diet		
Ingredients, % DM	Soybean meal	Linted cottonseed	Heated linted cottonseed
Alfalfa silage	33.3	33.3	33.3
Corn silage	16.7	16.7	16.7
High moisture ear corn	36.0	26.1	26.1
Soybean meal	12.0	6.8	6.8
Linted-cottonseed	...	15.0	...
Heated linted-cottonseed[1]	...	...	15.0
Minerals and vitamins	2.0	2.1	2.1
DM, %	50.2	51.1	51.3
NEL, Mcal/kg DM	1.64	1.68	1.68
Crude protein, % DM	16.2	15.6	15.9

[1]Linted-cottonseeds were brought to 146°C in a Jet Pro roaster and steeped for 30 min prior to cooling.

Table 2. Lactation performance.

Measurement	Treatment			SEM	$P =$
	Soybean meal	Linted cottonseed	Heated linted cottonseed		
DMI, kg/d	24.0	23.6	23.7	.5	.7
Milk, kg/d	38.1	38.3	40.3	1.0	.2
3.5% FCM, kg/d	37.6	37.6	39.5	.9	.3
Milk fat, %	3.45	3.41	3.42	.09	.9
Milk protein, %	3.24[a]	3.10[b]	3.00[b]	.04	.001
Fat yield, kg/d	1.30	1.30	1.36	.04	.5
Protein yield, kg/d	1.23	1.18	1.20	.03	.4
Weight gain[1], kg	36	51	49		
Gain in body condition score[2]	.2	.3	.4		

[1]Average weight at wk 27 and 28 minus average weight at wk 1 and 2 of the experiment.
[2]Body condition score at the end of the experiment minus beginning score. Range from 1 to 5, where 5 is the highest condition.

Milk Production and Blood Phosphorus Concentrations of Cows Fed Low and High Dietary Phosphorus

T.R. Dhiman, L.D. Satter and R.D. Shaver

Introduction

The National Research Council recommended dietary allowance of phosphorus (P) for a cow weighing 650 kg and producing 40 kg milk/day with 3.5% fat is .41 to .48% of dietary DM. However, many nutritionists recommend dietary levels of .5 to .6% P. Feeding excessive P not only increases feed cost but also creates problems with nutrient management on the farm.

The objective of the present experiment was to measure cow response to low and high dietary P (.39% and .65% dietary P-dry matter basis).

Materials and Methods

Forty-six mid to late lactation Holstein dairy cows were fed a pretrial diet for 10 days. At the end of the pretrial period, cows were blocked according to milk yield. Cows within blocks were assigned randomly to low P and high P treatments. Ingredient composition of the diets is in Table 1. Experimental diets were fed for 84 days. Cows were housed in a free stall barn and were fed as a group. The diets were fed as a TMR once daily and feed consumption and milk production were recorded. Monthly composite samples of silage and other feed ingredients were analyzed for phosphorus and calcium. Chemical composition of the TMR was calculated from chemical analysis of the individual feed ingredients. Blood samples were collected from the coccygeal vein or artery on three consecutive days from each cow at the end of the pretrial period, at wk 7 and wk 12 of the experiment. Inorganic phosphorus analysis was performed on blood serum samples.

Results and Discussion

Cows fed diets containing low and high P content had no effect on DM intake, milk yield, and milk composition in the present experiment (Table 2). Blood serum P concentrations of cows fed the low P diet were significantly lower compared with cows fed the high P diet at wk 7 and 12 of the experiment. As shown in Figure 1, concentrations of blood serum P in cows fed the low P diet were lower ($P = .06$) at wk 12 compared with P concentrations at wk 7 of the experiment. No change in serum P concentration was observed between wk 7 and 12 of the experiment in cows fed the high P diet (Fig. 1).

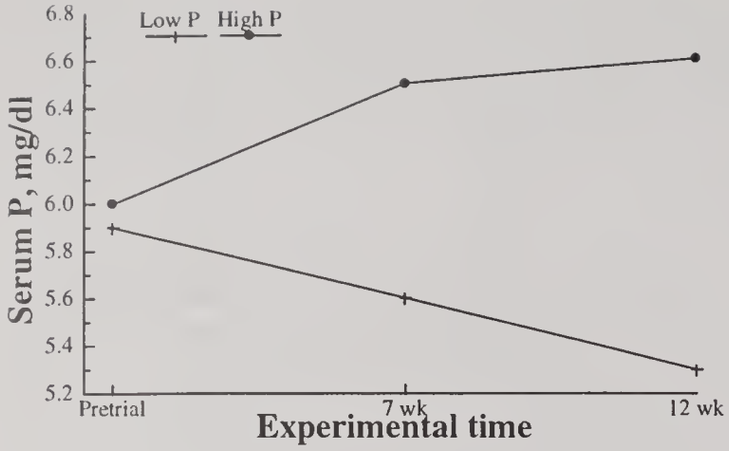

Figure. 1. Blood serum phosphorus concentrations of cows fed low and high phosphorus diets. (Each point represents an average of three samples taken from 23 cows during 3 days). SD was .62, .60, and .63 during prtetrial, 7 wk, and 12 wk, respectively.

Summary

Cow performance was not affected by feeding slightly lower amounts of P than recommended by NRC. However, concentration of blood serum P decreased slightly throughout the 84 day experiment. It is not known how much further, if any, blood serum P might decrease had the trial been longer. Longer term studies are being planned.

Table 1. Ingredient composition of diets.

	Treatment		
Ingredient	Pretrial diet	Low P	High P
Alfalfa silage	45.0	45.0	45.0
Corn silage	10.0	10.0	10.0
High moisture ear corn	18.70	19.0	18.15
Barley	12.0	12.0	12.0
Soybean meal	3.0	3.0	3.0
Roasted soybean	10.0	10.0	10.0
Dicalcium phosphate	0.15	0.15	0.15
Sodium mono-phosphate	0.30	0.0	0.85
Calcium carbonate	0.30	0.30	0.30
Trace-mineralized salt	0.50	0.50	0.50
Magnesium oxide	0.05	0.05	0.05
Vitamin ADE[1]	trace	trace	trace
Calcium, % DM	.59	.59	.59
Phosphorus, % DM	.48	.39	.65

[1]Vitamin supplement was added to supply vitamin A, 146,785; vitamin D, 48,928; and vitamin E, 489 IU/day per cow.

Table 2. Feed intake, milk yield, milk composition, and concentrations of blood serum P of cows fed diets containing low and high P content[1].

	Treatment			
Measurement	Low P	High P	SEM	*P*
DM intake[2], kg/d	22.0	22.1	-	-
Milk yield, kg/d	23.9	24.4	.5	.9
3.5% FCM, kg/d	24.9	25.2	.6	.3
Milk fat, %	3.88	3.97	.08	.4
Milk protein, %	3.48	3.60	.05	.3
Lactose, %	4.73	4.71	.04	.07
Blood serum P, mg/dl				
Pretrial	5.90	6.02	.13	.5
At wk 7 of expt.	5.64	6.52	.12	.01
At wk 12 of expt.	5.29	6.54	.12	.01

[1]Covariate adjusted LS Means.
[2]Cows were fed as a group; therefore no statistical comparison was made.

Dietary Effects on Conjugated Linoleic Acid Content of Cows' Milk

T.R. Dhiman, G.R. Anand, L.D. Satter and M. Pariza

Introduction

Conjugated linoleic acid (CLA) is an intermediate compound of biohydrogenation of linoleic acid in the rumen. Conjugated linoleic acid has been shown to have anticarcinogenic properties and possibly other effects that would be positive for human health. Two experiments were conducted to determine the content of CLA in milk of cows grazing pasture (Experiment 1) or those fed conserved forages (Experiment 2).

Materials and Methods

Experiment 1. Fifty-three cows were randomly assigned before calving to one of three treatment groups. Cows calved between March and May of 1995. From early May until the end of September cows grazed a permanent pasture (P) containing a mixture of grass and legume species. During the grazing season, cows in the three treatment groups consumed either 1/3, 2/3, or all of their daily feed from the pasture. The balance of feed for the 1/3 P and 2/3 P groups was supplied by a supplement. The supplement for the 1/3 P group contained (DM basis): alfalfa hay 25%, high oil high moisture ear corn (HOHMEC) 48.3%, soybean meal 6% and roasted soybeans 18%. The supplement for the 2/3 P group contained: alfalfa hay 50%, HOHMEC 28.4%, and roasted soybeans 18%. Milk samples were collected from 18 cows (6 in each group) during the month of August and from 35 cows (12, 11 and 12 cows in 1/3 P, 2/3 P, and P groups) during September. Milk samples were analyzed for fatty acid composition.

Experiment 2. Fifty-three cows were randomly assigned before calving to either normal corn (NC) or high oil corn (HOC) treatments. From wk 3 to 24 of lactation, cows in the NC treatment were fed diets containing normal corn silage and normal high moisture ear corn, whereas cows in the HOC treatment received high oil corn silage and HOHMEC. Diets contained (DM basis): alfalfa silage 27.5%, corn silage 22.5%, HMEC 31.5%, soybean meal 10%, roasted soybeans 4.6%, and blood meal 2%. After 10 weeks or more of feeding the experimental diets, milk samples were collected twice from 12 cows (6 in each treatment) and analyzed for fatty acid composition. Fatty acid profile of feed ingredients is in Table 1.

Results

The CLA and total unsaturated fatty acid contents in milk were higher in the P group compared with the 2/3 P and 1/3 P groups in experiment 1 (Table 2). In experiment 2, the fatty acid composition of the milk was similar for both groups. The CLA content was 3.7 and 4.0 mg/g of milk fat in the normal corn and high oil corn groups, respectively.

Summary

Milk from cows grazing pasture had higher CLA content than milk from cows fed conserved diets containing 50:50 forage and grain.

Table 1. Fatty acid composition of feed ingredients (Fatty acid, mg/g fat).

Ingredient	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	Others
Alfalfa hay	7.3	251.6	32.1	26.1	150.2	230.2	302.6
Alfalfa silage	12.2	172.4	28.8	45.5	202.3	301.8	237.0
Normal corn silage	6.4	142.8	25.9	212.8	487.8	41.6	82.7
High oil corn silage	2.7	123.5	29.9	254.0	470.5	25.7	93.6
Pasture grass	9.9	132.2	23.9	34.0	159.1	410.8	230.0
NHMEC	2.2	120.6	19.4	264.2	537.9	14.7	41.1
HOHMEC	.6	109.4	24.1	312.2	461.9	9.4	82.4
Soybean meal	2.5	151.6	43.4	136.9	524.7	90.1	50.9
Roasted soybeans	1.4	104.5	40.8	224.4	512.6	85.3	31.0
Blood meal	30.4	221.6	166.9	244.5	97.8	9.8	229.0

Table 2. Fatty acid composition of the milk (mg/g of fat)[1].

	Experiment 1					Experiment 2			
Fatty acid	1/3P	2/3P	P	SEM	*P*	NC	HOC	SEM	*P*
C10:0	20.3	18.9	18.9	1.5	.6	30.3	26.7	.7	.2
C:12:0	24.5	23.0	21.9	1.0	.09	40.8	34.7	.9	.2
C14:0	91.0	91.0	88.4	2	.08	130	119	2	.3
C16:0	243	245	251	5	.2	330	302	7	.5
C:16:1	12.6[b]	13.1[b]	18.0[a]	.5	.02	17.1	14.7	.7	.5
C18:0	153	159	123	6	.07	93	118	3	.2
C18:1	303[b]	311[b]	331[a]	7	.001	203	232	5	.4
C18:2	42.7[a]	31.4[b]	16.6[c]	1	.003	35.0	35.6	.9	.15
CLA[2]	8.4[c]	13.7[b]	22.7[a]	.9	.0002	3.7	4.0	.2	.9
C18:3	8.2[c]	13.5[b]	16.5[a]	.2	.001	7.6	7.4	.1	.5
Others	93	80	92	7	.4	109	106	2	.9

[1]Means in the same row with different superscripts differ (*P* as indicated).
[2]Conjugated linoleic acid (cis 9, trans 11).

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R.P. Walgenbach - Farm Manager

The 1995 growing season began with very cold and fairly wet conditions in early April. Rainfall recorded at the farm entrance rain gauge in inches were 2.68 in April, 2.73 in May, 1.90 in June, 4.24 in July, 4.58 in August, 1.32 in September and 2.71 in October. The cold spring was followed by hot and dry conditions in June. Very hot temperatures continued in July and August. Temperatures were near or exceeded 90 degrees F on several days and a few days we recorded temperatures of 100°F or higher. Several of these hot days occurred during critical corn pollination times for several fields. A lack of snow cover in late January exposed the alfalfa crop in this region to risk of winter injury, but fortunately moderate air temperatures during this period did not cause much injury to most alfalfa stands. The alfalfa survived the winter season in reasonably good condition with stands similar to those harvested in 1994, but initial spring growth was delayed and slow due to cold temperatures.

This year we planted 96 acres of barley, 212 acres of soybeans, 359 acres of corn, 35 acres of spring seeded alfalfa and 93 acres of summer seeded alfalfa. Barley planting started on 24 April and was completed on 2 May. All barley was no-till planted at 100 pounds per acre into soybean stubble with a John Deere 750 no-till drill. A 31 acre field was surface tilled with an Aer-Way tillage implement prior to seeding barley. Approximately 5,000 gallons per acre of liquid manure were spread on all barley fields. Alfalfa was no-till seeded on 12 May after spraying fields with 2 quarts per acre of roundup. These fields had significant amounts of quackgrass and the cold spring temperatures produced insufficient growth for early treatment with roundup. Varying rates of roundup were sprayed on harvested barley fields followed by no-till seeding of alfalfa from 11 to 16 August at a rate of about 13 pounds per acre. Corn was planted at about 33,000 seeds per acre from 1 to 22 May. About 35 acres were planted following conventional tillage, 105 acres following one pass with dyna drive tillage and 245 acres were planted no-till. Most corn ground, except autumn killed alfalfa, had about 9,000 gallons per acre of liquid manure. All corn received 100 pounds per acre of 10-20-20 starter fertilizer and 160 pounds per acre of nitrogen from a combination of soybean, alfalfa and manure nitrogen credits and surface applied 28% liquid nitrogen (N) fertilizer. Soybeans were no-till seeded at about 225,000 seeds per acre from 2 to 19 May.

Barley yields averaged 59.5 bushels per acre and ranged from 44.1 bushels per acre for a field of foundation seed barley to 78.4 bushels per acre for malting barley. Soybeans produced an average yield of 59.6 bushels per acre and ranged from 49.7 to 64.9 bushels per acre. Soybean harvest occurred from 29 September to 12 October. The cold soil conditions delayed and prevented emergence of early planted soybeans which contributed to significant stand loss and less than optimal stands. White mold symptoms were evident in all soybean fields; however, with one exception, it was less severe than that observed in 1994. One 39 acre field had several pockets with 40 to 50% severely infected plants.

About 1493 wet tons of corn silage were harvested from 84.5 acres between 6 to 22 September. Yields ranged from 5.6 to 8.0 tons dry matter per acre and averaged 6.5 tons per acre. We harvested 250 acres of high moisture ground ear corn from 9 September to 17 October. The yields, adjusted to a 29% moisture level, ranged from 3.6 to 5.4 tons per acre

and averaged 5.0 tons per acre. The shelled corn yield equivalent for 5.0 tons per acre of high moisture ground ear corn at 29% moisture is approximately 138 bushels per acre. Twenty-four acres of corn harvested as dry shelled corn yielded 153.4 bushels per acre. The dry conditions during June, excessive heat throughout much of the summer, and the worst corn borer infestation in Wisconsin history decreased yield expectations, especially following the record yields from the 1994 season. The harvested alfalfa from established fields yielded an average of 4.24 tons dry matter per acre and ranged from 3.2 to 5.4 tons dry matter per acre. The cold spring delayed regrowth initiation and slowed subsequent growth and caused reduced first harvest yields. The second crop yields suffered from lack of moisture and the summer heat affected both second and third crops. A field of Marathon red clover harvested on 19 June, 25 July and 31 August produced 5.1 tons dry matter per acre. The spring and early summer growth benefited from cool temperatures and produced a first crop yield of 2.8 tons dry matter per acre.

Two fields were grid-mapped and soil sampled using Global Positioning System (GPS) technology this spring. A local cooperative spread lime at a variable rate on one field this autumn. We plan to explore cautiously this emerging technology to ensure that our investment in this technology is practical and profitable.

The conversion of the gate opening signal from microwave transmission to telephone line transmission was completed in early spring. This has improved the operation and reliability of the automated gate; however, we continue to have problems with the system. The modems communicating between computers at the gate 8 entrance and the manned station at gate one have been very susceptible to lightening strikes and electrical surges. We have been working with Wisconsin Power and Light and personnel at Badger Army Ammunition Plant to resolve this problem.

The Center purchased a John Deere Model 9500 combine, a New Holland Model 2450 self-propelled mower/conditioner and a used Owatonna stack maker. With the equipment purchases planned for 1996, we should complete the majority of our equipment purchases to replace previously leased equipment. The Center also added a 32 X 124 foot bunker silo and increased the size of an existing bunker to 32 X 124 feet. This expansion is needed to provide additional feed for the herd expansion and to give us a modest carryover of feed in surplus crop years.

Again this past season we have had several construction projects and disruptions and the usual but unanticipated breakdowns that cause frustrations and changes in operations. I appreciate the cooperation and diligence of our employees in working with these situations. I thank all of them for their past and continued efforts toward accomplishing our research objectives.

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Annual Dairy Operations Report
January 1996

L.L. Strozinski - Herd Manager

Herd Statistics

Herd Inventory		
Milking cows		300
Dry cows		35
average cow age	45 months	
percent first lactation	46%	
percent second lactation	21%	
percent third lactation	14%	
percent greater than third	17%	
Herd replacements		320
Total		***655***
Herd Performance		
Cows calved		366
Heifer calves born	177 live	12 dead
Bull calves born	188 live	23 dead
Heifer calves died < 1 year old		2 (1.13%)
DHIA rolling herd average		
milk		19,908 lbs.
protein		728 lbs.
fat		621 lbs.
Milk sold in 1995		6,225,278 lbs.
Heifer calves sold		14
Bull calves sold		188
Cows sold		143
Cows culled for:		
reproduction problems		42
poor production		21
poor udder		19
poor feet and legs		18
mastitis		12
injury		5
other		13
Cattle sales revenue		$69,200.00
Herd Reproduction		
Average days open		114
Average calving interval		12.75 months
Average services per conception		2.5
Average age at first calving		25 months

Although 1995 was a good year overall at the farm, progress with the herd in terms of growth and performance was not as good as it has been in past years. Probably the largest contribution to this slowed performance was the extended hot weather of the 1995 summer. Similar to the associated heat stress problems faced by nearly all midwest herds, our herd suffered considerable setbacks in milk production and reproductive performance.

Overall culling from the herd, especially that which was due to failed reproductive performance, was high in 1995. Consequently, herd numbers have not grown as much as in previous years and the average age of our milking herd has decreased to 45 months. Forty-six percent of the cows in the present milking herd are first calf heifers. This compares to 38 percent a year ago. On a strong note, our heifer crop looks very nice, have especially good udders and are producing very well.

Herd reproductive performance suffered during the hot summer. Normally 30 to 40 cows calve in our herd each month. Because of hot summer heat, we have only 15 to 20 cows due to calve in April and May, respectively. Recovery from the heat stress did not occur until October and resulted in 64 animals due to calve in August 1996.

Declining cull cattle prices and extremely low bull calf prices in 1995 caused our cattle sales revenue to fall short of projections. It was not uncommon to receive less than ten dollars for a small bull calf that would have brought sixty dollars a year ago.

The economic climate dictated continued emphasis on farm cost reductions and overall streamlining of the operation while continuing the necessary research support. Numerous activities were reevaluated, modified or discontinued to facilitate a reduction of one full time employee in the dairy operation. In 1995, twelve dairy nutrition experiments were conducted which involved more than 350 cows.

Continued emphasis is being placed on expanding the milking herd to 320 cows. In late summer a concrete feed pad, manure handling ramp, fence line feeding system and a cattle mound were added near our hay storage building which had been previously modified for cattle housing. This facility can now house 70 to 80 animals in the winter as well as provide feed bunk space for summer pasture supplementation.

A significant management change made in 1995 was the implementation of a tail docking program for the herd. The tails of all USDFRC cattle were removed to promote cow and facility cleanliness and ultimately produce a cleaner product. We have been very satisfied with this measure and are seeing a modest decline in somatic cell counts and cases of mastitis. We have also found that tail docking has reduced our bedding material and labor costs significantly. Continued efforts have been made to improve cow comfort as well. During the summer, misters were installed near the feed bunks in the free stall barn to facilitate cattle cooling. Currently we are in the process of modifying the free stalls by relocating the dividers and neck rails and by opening the stall fronts to provide more air flow and lunge space for the cows.

A milk cooling project is underway at the farm. A consortium of Wisconsin power companies, Dairy Equipment Company and the University of Wisconsin Milking Laboratory are working together to evaluate an energy saving system for milk cooling. Well water is used in the first stage of a plate cooler to cool the milk before being used for drinking water by the herd. In addition, a glycol solution cooled by outdoor ambient temperature is used in a second stage of the plate cooler. During the winter, milk enters the bulk tank at 41 degrees which eliminates the need for the bulk tank compressors. We expect this project to significantly cut our milk cooling costs and provide valuable information to dairy producers in cool climates.

I am happy to announce that in 1995 one of our cows was classified as excellent by the Holstein Association. She is number 2818, DFR Brass Bell Caterina. She is a four year old sired by Schutzs Brass Bell. Her breakdown is E E V V E - 90.

Special thanks to the entire farm staff for their dedication and hard work during these more challenging economic times. Special thanks also to our research staff for their understanding and willingness to cooperate as much as possible to minimize overall farm costs.

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